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CONTENTS OF VOLUME XXXV.

No. 1, July, 1918.

Steenbock, H. Isolation and identification of stachydrin from alfalfa hay 1

Bernhard, Adolph. The determination of cholesterol in blood serum 15

Osborne, Thomas B., and Mendel, Lafayette B. The choice between adequate and inadequate diets, as made by rats 19

Harding, Victor John, and Fort, Charles Atherton. The amino-acids of mature human placenta 29

Addis, T., and Shevky, A. E. The rate of color production in alkaline solutions of dextrose and picrate 43

Addis, T., and Shevky, A. E. A modification of the picrate method for blood sugar determinations 53

Steenbock, H., Kent, Hazel E., and Gross, E. G. The dietary qualities of barley 61

Baumann, L., and Hines, H. M. The origin of creatine. III 75

Witzemann, Edgar J. The effect of variations in the available alkali on the yield of acetone in the oxidation of butyric acid with hydrogen peroxide 83

Denis, W., and Minot, A. S. Ammonia excretion as influenced by the ingestion of alkalies 101

Bosworth, A. W., and Giblin, Louise A. The casein of human milk 115

Haas, A. R. C. On the preparation of ovalbumin and its refractive indices in solution 119

Van Slyke, Lucius L., and Baker, John C. The preparation of pure casein 127

Baker, John C., and Van Slyke, Lucius L. A method for making electrometric titrations of solutions containing protein 137

Van Slyke, Lucius L., and Baker, John C. Free lactic acid in sour milk 147

No. 2, August, 1918.

Dernby, Karl Gustav. A study on autolysis of animal tissues 179

Wells, H. Gideon. The purine metabolism of the Dalmatian coach hound 221

Bock, Joseph C., and Benedict, Stanley R. A new form of colorimeter. Plates 1 to 3 227

BERMAN, LOUIS. A rapid method for the determination of iron in small quantities of blood 231
OSTERHOUT, W. J. V. The determination of buffer effects in measuring respiration 237
GIVENS, MAURICE H. Studies in calcium and magnesium metabolism. V. Further observations on the effect of acid and other dietary factors 241
CLARK, GUY W. The properties and composition of oocytin 253
BAUMANN, LOUIS, and INGVALDSEN, THORSTEN. Concerning histidine and carnosine. The synthesis of carnosine 263
BAUMANN, LOUIS, and INGVALDSEN, THORSTEN. An oxidation product of creatine 277
LEVENE, P. A., and TAYLOR, F. A. Reduction of aldehydes to corresponding alcohols. I. Reduction of heptylic aldehyde (oenanthol) 281
LEVENE, P. A., and WEST, C. J. Cephalin. V. Hydrocephalin of the egg yolk 285
MATSUO, IWAO. Formation of the unsaturated acids in the animal organism. First paper. The behavior of *p*-methoxyphenylpropionic acid in the organism of the rabbit 291
DAUGHTERS, MILO REASON. The food value of eulachon 297
SHERMAN, H. C., and WINTERS, JET C. Efficiency of maize protein in adult human nutrition 301
SOMMER, H. H., and HART, E. B. Effect of heat on the citric acid content of milk. Isolation of citric acid from milk 313
KOTAKE, YASHIRO. The formation of oxyphenyllactic acid in the animal organism and its relation to tyrosine catabolism 319
MATSUOKA, ZENJI. The relation between nutrition and the formation of kynurenic acid from tryptophane 333
MORI, YOSHITANE. The decomposition of muconic and adipic acids in the animal body 341
MCCLUGAGE, HARRY B., and MENDEL, LAFAYETTE B. Experiments on the utilization of nitrogen, calcium, and magnesium in diets containing carrots and spinach 353
HART, E. B., and HUMPHREY, G. C. The relation of the quality of proteins to milk production. IV 367
WEBER, F. C., and WILSON, J. B. A method for the separation and quantitative determination of the lower alkylamines in the presence of ammonia 385
FISKE, CYRUS H. The inhibition of foaming 411

No. 3, SEPTEMBER, 1918.

KOESSLER, JESSIE HORTON. Studies on pollen and pollen disease. I. The chemical composition of ragweed pollen 415
COHEN, BARNETT, and MENDEL, LAFAYETTE B. Experimental scurvy of the guinea pig in relation to the diet 425

Crozier, W. J. On indicators in animal tissues..... 455
Greenwald, Isidor. Observations on the significance of glycollic acid, glyoxal, glycol aldehyde, and amino-aldehyde in intermediary metabolism..... 461
Sato, Akira. A rapid micro method for the determination of phosphate and total phosphorus in urine and stools..... 473
Hess, Alfred F., and Unger, Lester J. The scurvy of guinea pigs. I. The experimental dietary..... 479
Hess, Alfred F., and Unger, Lester J. The scurvy of guinea pigs. II. Experiments on the effect of the addition of fruits and vegetables to the dietary..... 487
Loeb, Jacques. The influence of electrolytes upon the osmotic pressure of gelatin solutions..... 497
Rappleye, W. C. A simple application of the Volhard principle for blood plasma chlorides..... 509
Denis, W. The determination of creatinine and creatine in blood... 513
Steenbock, H., Boutwell, P. W., and Kent, Hazel E. Fat-soluble vitamine. I..... 517
Hatai, Shinkishi. The refractive index of the blood serum of the albino rat at different ages..... 527
McCann, William S. A study of the carbon dioxide-combining power of the blood plasma in experimental tetany..... 553
Egerer, Grete. A modified method for the preparation of picramic acid..... 565
Lewis, Julian H. The metabolism of glycine given intravenously at constant rates..... 567
Index to Volume XXXV..... 577

ISOLATION AND IDENTIFICATION OF STACHYDRIN FROM ALFALFA HAY *

By H. STEENBOCK.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

(Received for publication, May 9, 1918.)

In 1912 an experimental inquiry into the character of the water-soluble nitrogen of alfalfa hay by Dr. R. H. Carr, while a student in this laboratory, suggested the presence of a nitrogenous compound which caused discrepancies in the values obtained for histidine and lysine by the Van Slyke method as compared with the Kossel method. In view of the importance which individual amino-acids have assumed in the biological evaluation of feeding materials, and especially in view of the fact that there has been a tendency to apply these methods unreservedly and indiscriminately to plant materials, an attempt has been made to obtain definite information in regard to the nature of the compound here concerned. As a result stachydrin, a pyrol derivative, has been isolated and identified.

With a full realization of the fact that the absence or deficiency of a ration in certain amino-acids may be responsible for nutritive failure, there has come a demand for a chemical method for determining accurately the amounts of the different protein cleavage products present. The determinations must be fairly accurate or nutritive values forecast on such data will not approximate results obtained in actual feeding practice.

The Van Slyke and Kossel methods were developed to be applied to the cleavage products of pure proteins. Unfortunately, so far it has not been possible to accumulate quantitatively the proteins or their cleavage products occurring in feeds in a form free from carbohydrates. This field of endeavor is apparently

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beset with many difficulties, not only because of the solubility and difficult precipitability of the amino-acids themselves, but because many plant materials contain carbohydrates which are dissolved and precipitated with the proteins by the usual reagents. As a result, attempts have been made to hydrolyze the proteins in the presence of the accompanying carbohydrates and then to determine certain amino-acids, but with different degrees of success.[1–3] The possibilities for condensations and other interreactions between the amino-acids and compounds found in the hydrolysis are so numerous as to open undoubtedly to serious question the reliability of the data obtained.[4–6]

To the above difficulty must now be added the possibility of erring by taking for granted that all the nitrogen in the feeding materials is present in the form of proteins or their cleavage products. To be sure, this assumption is actually made when crude protein is calculated on the basis of the total nitrogen content. In general, however, the percentage of error in the total protein content with respect to nutritive value can be but small, but when the entire error falls on one particular indispensable constituent of the ration and that in itself is present in but small amounts, the error may become a very serious one indeed.

In the Kossel method of dissection of the diamino-acid fraction, lysine is isolated and determined as the picrate. It so happens that stachydrin picrate though not quite as insoluble as lysine picrate yet is sufficiently insoluble to lead the uninitiated to assume that lysine alone was being determined.

In the Van Slyke method of analysis, as stachydrin does not yield its nitrogen to nitrous acid treatment, the stachydrin nitrogen would be calculated as non-amino nitrogen belonging to histidine. The histidine value being incorrect the value for lysine

[1] Grindley, H. S., Joseph, W. E., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 1778. Grindley, H. S., and Slater, M. E., *ibid.*, 2762.

[2] Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.

[3] Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii, 477. Hart, E. B., and Sure, B., *ibid.*, 1916–17, xxviii, 241.

[4] McHargue, J. S., *J. Agric. Research*, 1918, xii, 1.

[5] Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630. Gortner, R. A., *J. Biol. Chem.*, 1916, xxvi, 177. Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1917, xxxix, 2477.

[6] Roxas, M. L., *J. Biol. Chem.*, 1916, xxvii, 71.

would also be incorrect as this is obtained by difference of the arginine and histidine values from the total nitrogen.

Blish[7] has indicated that in wheat flour there are present forms of nitrogen not to be classed with any of the cleavage products of proteins. This does not seem so very remarkable when it is considered that bases and alkaloids are found in considerable amounts in many plants, but ordinarily the biological chemist has not taken cognizance of the probability of their presence in those plant materials commonly used for food.

Stachydrin undoubtedly is one of many similar nitrogen-containing compounds to be found in the water-soluble fraction of feeds. It does not occur in large amounts in alfalfa, 350 gm. yielding 0.875 gm. of the hydrochloride, which is equivalent to 0.068 gm. of nitrogen. This represents 0.5 per cent of the total nitrogen of the hay. In per cent of the total nitrogen of the hay, when analyzed by the Kossel method, the value for lysine nitrogen would be 0.5 per cent too high; by the Van Slyke method it would be 0.25 per cent too low and the histidine nitrogen 0.75 per cent too high. Except for their biological significance these amounts in themselves may not appear very significant. In all cases they are to be looked upon as minimum values as the method used for the determination of stachydrin—isolation and crystallization of the hydrochloride—is far from being quantitative. The results may be from 10 to 15 per cent too low.

EXPERIMENTAL.

Preparation I.

Alfalfa hay, free from weeds and unbleached, was extracted repeatedly with boiling water until the final extract was but faintly colored. This was best done by packing the hay in a muslin sack and immersing the whole in a crock half filled with water into which live steam was passed for a period of 3 hours. By repeating the extractions, 80 liters of extract were obtained from about 13.5 kilos of hay. The extract was cooled and then precipitated with a saturated solution of tannin. Great difficulty was experienced in filtering this as the precipitate was too light and too colloidal in character to separate by centrifugating or to allow filtration through coarse filters. After various trials with additions of different

[7] Blish, M. J., *J. Biol. Chem.*, 1918, xxxiii, 551.

salts, acids, and alkalies it was found that sodium hydroxide added to slight alkalinity—which was indicated by the extract turning a bright yellow—would flocculate the suspended precipitate so that it settled rapidly and filtered out completely. To the clear liquors lead acetate was added to complete precipitation, filtered, and the excess of lead removed with hydrogen sulfide at the boiling temperature.

The filtrate was concentrated to about 16 liters volume, then cooled, acidified to the extent of 5 per cent sulfuric acid, and precipitated with a 10 per cent solution of phosphotungstic acid in 5 per cent sulfuric acid till immediate precipitation ceased. After 12 hours the precipitate was filtered off and washed thoroughly with dilute phosphotungstic acid in 5 per cent sulfuric acid solution. It was then triturated in a large mortar with a small amount of water until well suspended, and hot barium hydroxide solution added, with continued trituration, until strong permanent alkalinity obtained. The temperature of the mixture was not allowed to go above 40° for fear of decomposing some of the bases, and care was taken to have an excess of barium hydroxide present as the bases liberated from phosphotungstate precipitates often are strongly alkaline in reaction themselves. The barium phosphotungstate and sulfate were removed by filtration on a Buchner funnel and washed thoroughly with hot water. From the combined filtrates and washings excess of barium hydroxide was removed with carbon dioxide at the boiling temperature. The filtrate on evaporation to dryness left a semicrystalline mass of a dark brown color and a strong alkaline reaction. A small test portion gave a slight precipitate with ammoniacal silver nitrate solution which indicated the probable presence of traces of purines. The preparation was accordingly taken up in water, slightly acidified with nitric acid (freed from nitrous acid by boiling), and silver nitrate added till a test portion gave a brown precipitate with barium hydroxide. A small precipitate resulted which was allowed to settle and filtered off on a folded filter. The filtrate was then made strongly alkaline with barium hydroxide, and the precipitate consisting of small amounts of the histidine-arginine fraction removed by filtration. Barium was removed with carbon dioxide, traces of silver with hydrogen sulfide, and the filtrate was again precipitated with phosphotungstic acid to free the bases from nitrate. The phosphotungstates were decomposed as before, and the semicrystalline mass was again obtained upon evaporation of the filtrate after the removal of the barium. The character of the alkaline residue and method of preparation suggested that here some substance similar to lysine was present. Like lysine, it absorbed carbon dioxide from the air—which was liberated upon the addition of acids—and was strongly alkaline in reaction. A number of salts were prepared for identification.

Picrate.—The picrate was made by the addition, drop by drop, of a strong alcoholic solution of picric acid to a few gm. of the residue taken up in the minimum amount of alcohol. Addition

of precipitant was discontinued as soon as precipitation ceased because a small test portion showed that resolution occurred upon the addition of an excess. Precipitation from water solution by an alcoholic solution of picric acid was also found possible, but there is always danger of a slight excess of picric acid being precipitated as it is less soluble in water than in alcohol. After 12 hours the crystals were examined under the microscope and found to consist, not of needles, as in the case of lysine, but of coarse feather-formed crystals with occasional prisms in star-shaped aggregates. This latter form was found to be formed upon slow crystallization. In no case were any needles obtained either from alcohol or water solution. They melted unsharply with decomposition, as is usual for picrates, at about 188°.

Hydrochloride.—The picrate was taken in solution in water, strongly acidified with hydrochloric acid, and shaken repeatedly with ether until entirely free from picric acid. The hydrochloric acid solution was then evaporated to dryness and the residue found to be very soluble in methyl alcohol, from which it could be precipitated by ether in leafy crystals. While the first fraction thus obtained was highly colored, succeeding ones were snowy white, but this method of purification resulted in such large losses that it was not deemed practical. Previous decolorization with animal charcoal was found very inefficient.

Chloroplatinate.—A small amount of the hydrochloride residue, obtained by evaporation of the above ether-alcohol solutions was taken in solution in alcohol on a slide and treated with a drop of an alcoholic solution of platinum chloride. A precipitate of microscopic needles formed at once. The entire hydrochloride residue was then taken up in alcohol and precipitated. After 24 hours the crystalline precipitate was removed on a suction filter, washed rapidly with cold 95 per cent alcohol, and then recrystallized from 50 per cent alcohol. The salt separated as beautiful plates and octahedrons. Dried at 101–102° it failed to give a sharp melting point,—decomposition setting in at about 200°. Upon incineration 0.1394 gm. yielded 0.0386 gm. of metallic platinum equivalent to 27.69 per cent of its weight.

Preparation II.

9 kilos of alfalfa hay were extracted with water as before, and the extract was precipitated directly with basic lead acetate. The filtrate freed from lead with hydrogen sulfide was evaporated to a small volume for extraction with alcohol, but all attempts to secure a dry residue for direct extraction were futile. Accordingly the phosphotungstic precipitation and the subsequent removal of purines, histidine and arginine were carried out as before. The product was again a brown, strongly alkaline reacting, semi-crystalline mass. It was taken up with a saturated solution of picric acid in alcohol and then carefully, but completely, precipitated by the further addition of this reagent. After 24 hours the precipitate was filtered off and recrystallized from hot water. The crystals were found to have the same crystalline form as those of the first preparation. That the picrate does not lend itself to purification of the bases was realized upon examination of this crystalline mass under the microscope. Numerous small brown masses of pigment were found to be scattered among the crystals even after recrystallization from a clear solution.

The moist picrate obtained as above and weighing about 7 gm. was transferred to a separatory funnel with dilute hydrochloric acid and freed from picric acid with ether as before. The impurity of the picrate became even more apparent as the solution of the hydrochloride was brown in color and drops of tarry substances deposited themselves on the sides of the funnel. They were carefully removed by filtration and the solution boiled up repeatedly for hours with animal charcoal until almost colorless. On evaporation to dryness a slightly colored mass of white crystals remained. They were taken up in water and boiled up thoroughly with animal charcoal to effect further decolorization. After repeating this process twice—boiling out the charcoal with water thoroughly each time—on evaporation a crystalline residue but very slightly colored remained. Drying at 98° for $2\frac{1}{2}$ hours caused its color to deepen slightly, but this treatment was found necessary to remove all water and excess of hydrochloric acid.

This crystalline mass was found to be almost completely soluble in absolute alcohol. Only a faint turbidity which was readily removable by filtration indicated the presence of some other compound. The alcoholic solution on standing over night deposited a number of small crystals at its upper edge in the beaker which suggested the possibility of purification by recrystallization from this solvent. The solution was therefore concentrated to 10 cc. volume on a sand bath. On standing, a crop of beautiful snow-white crystals was obtained. They were filtered off with suction, washed with cold absolute alcohol, and then dried at 98°. This treatment did not cause any darkening of the preparation.

It was found to melt sharply with decomposition at 211.5° (uncorrected). The filtrate was concentrated and a second crop of crystals obtained which likewise melted at 211.5° (uncorrected). The two fractions were therefore united giving a total of 1.1698 gm. of material. An ultimate analysis gave the following results:

Chlorine by Direct Precipitation.

0.0908 gm. hydrochloride gave 0.0706 gm. AgCl = 19.2 per cent chlorine.
0.0870 " " " 0.0671 " AgCl = 19.02 " " "

Nitrogen by Kjeldahl.

0.0725 gm. hydrochloride gave 0.0525 gm. N = 7.24 per cent nitrogen.
0.0806 " " " 0.0575 " N = 7.13 " " "

Carbon and Hydrogen by Combustion.

0.1300 gm. hydrochloride gave 0.2244 gm. CO_2 = 47.14 per cent carbon.
0.0902 " H_2O = 7.71 " " hydrogen.

Determination of Properties.

Stability to Alkalies.—0.1616 gm. of the hydrochloride was dissolved in 25 cc. of water, 12.5 gm. of potassium hydroxide were added, and boiled for 6 hours under a reflux. The condenser was then disconnected, 100 cc. of water were added, and slowly boiled down to the original volume. After cooling the residual liquors, 35 cc. of sulfuric acid together with a little copper sulfate were added, and then boiled as in the regular Kjeldahl nitrogen digestion. On making alkaline and distilling, 0.0119 gm. of nitrogen equivalent to 7.35 per cent of the weight of hydrochloride taken was distilled over. Compared with the previous determination of the nitrogen content which gave the average value of 7.18 per cent it is seen that no nitrogen is lost in the treatment with alkali. That very strong alkalies, approaching an alkaline fusion, are able to decompose the compound was demonstrated by evaporating a small test portion with concentrated alkali to dryness. As a state of fusion was approached, decomposition with the evolution of a methylamine odor set in. In view of these results it seemed very probable that a base with a methylated nitrogen atom in a ring structure characterized the compound under investigation.

Stability to Nitrous Acid.—0.1162 gm. of the hydrochloride was dissolved in 7 cc. of water and transferred with an additional 7 cc. of water to the deaminizing chamber of a Van Slyke apparatus. Nitrous acid was generated in the usual way and allowed to react upon the hydrochloride for 4 minutes with continued shaking. No nitrogen was liberated in this period of time. The negative result indicated the absence of a primary amino group. This was substantiated by the failure to obtain a typical isonitrile reaction.

Pyrol Reaction.—A small amount of the hydrochloride heated in a test-tube gave the characteristic pyrol red with a pine splinter moistened with hydrochloric acid.

Precipitation Reactions.—A small amount of hydrochloride in solution was found to precipitate with phosphomolybdic acid and with iodine in potassium iodide solution, but not with bromine water or with Millon's reagent.

Solubility in Chloroform.—As many alkaloids and plant bases are soluble in chloroform, a small amount of the hydrochloride was made strongly alkaline with sodium hydroxide and shaken out with this solvent Evaporation of the chloroform failed to show that any material had been extracted.

From the data accumulated up to this point it seemed very probable that stachydrin, first isolated by von Planta[8] in 1890 from *Stachys tuberifera* and later studied by von Planta and Schulze,[9] Jahns,[10] Yoshimura and Trier,[11] Schulze and Trier,[12] and Engeland[13] was the compound in question. Like stachydrin this compound is insoluble in chloroform, contains the pyrol ring, is decomposed by alkaline fusion with a liberation of methylated amine, contains no primary amino nitrogen, is precipitated by phosphotungstic acid, phosphomolybdic acid, and potassium triiodide, forms a crystallizable picrate, hydrochloride, and chloroplatinate, and is not precipitated by tannin, lead acetate, or by silver in either alkaline or acid solution. A comparison of

[8] von Planta, A., *Ber. chem. Ges.*, 1890, xxiii, 1699.

[9] von Planta, A., and Schulze, E., *Ber. chem. Ges.*, 1893, xxvi, 939.

[10] Jahns, E., *Ber. chem. Ges.*, 1896, xxix, 2065.

[11] Yoshimura, K., and Trier, G., *Z. physiol. Chem.*, 1912, lxxvii, 290.

[12] Schulze, E., and Trier, G., *Z. physiol. Chem.*, 1910, lxvii, 59.

[13] Engeland, R., *Arch. Pharm.*, 1909, ccxlvii, 463; *Z. physiol. Chem.*, 1910, lxvii, 403.

the analyses at hand with that calculated for stachydrin salts will bring out a comparison, even if not absolute agreement, in elementary composition.

	C	H	N	Cl
	per cent	*per cent*	*per cent*	*per cent*
Calculated for stachydrin hydrochloride	46.78	7.79	7.79	19.75
Found	47.14	7.71	7.18	19.13

	Pt
	per cent
Calculated for stachydrin chloroplatinate	28.02
Found	27.69

The discrepancies between the above analyses and the calculated values were undoubtedly due to lack of purity of the preparations as the differences were larger than one would expect from analytical errors. For further confirmation it became necessary to prepare more of the base and its salts.

All preparations were made by essentially the same procedure as followed in making Preparation I, but the method of purifying the hydrochloride in an attempt to secure a better product was modified. One difficulty which proved very troublesome was the formation of a certain amount of pigment which seemed to increase as the material was heated. An attempt was made to avoid this by concentrating the free base, previous to the formation of the hydrochloride, *in vacuo* below 60° but with no success; the product was as highly colored as before. Recourse was had to repeated boiling with animal charcoal to remove this pigment.

In previous preparations no attempt had been made to secure a better product by dividing up any of the salt preparations into successive fractions by crystallization or precipitation. It was thought probable that the chloroplatinate would be suitable for this, using crystal form as a criterion of purity, but this was seen to be impossible as, varying with the conditions, the chloroplatinate will crystallize in needles, plates, or octahedrons.

After a number of attempts with the chloroplatinate and the picrate the chloride accumulated from a number of preparations was used instead. It was boiled up repeatedly with animal charcoal until the solution was practically water-clear, then it was evaporated to dryness, and taken up in absolute alcohol. Upon concentration on a sand bath three fractions weighing re-

spectively 2.741, 2.954, and 1.097 gm. were obtained. They were identical in appearance microscopically and melted at 210–211° (uncorrected). Portions of the second fraction were used for nitrogen determinations. These determinations were made by combustion as many plant bases give off their nitrogen as ammonia in the Kjeldahl process with great difficulty.

I. 0.2662 gm. gave 1.92 cc. N at 20.5° and 747 mm. = 0.02146 gm. N or 8.06 per cent N.

II. 0.1909 gm. gave 1.38 cc. N at 20.5° and 747 mm. = 0.01542 gm. N or 8.08 per cent N.

Calculated for stachydrin hydrochloride, N= 7.79 per cent.

Fractional crystallization of the hydrochloride as indicated by the nitrogen content is apparently able to give a good preparation. Still better results in crystallization of the hydrochloride were obtained after a preliminary precipitation with a solution of mercuric chloride in alcohol. For this purpose the hydrochloride was dissolved in 95 per cent alcohol and the reagent added to complete precipitation. The precipitate collected as a heavy oil which failed to solidify on standing at 0°. The supernatant liquors were decanted and the residue was digested repeatedly with 95 per cent alcohol at 60°. Finally it was heated on the steam bath to remove all alcohol, taken up in boiling water, and the mercury removed with hydrogen sulfide. The filtrate was decolorized with animal charcoal, evaporated to dryness, and the residue recrystallized from absolute alcohol. A perfectly white, beautifully crystalline preparation melting at 211–215° (uncorrected) was obtained. It was dried *in vacuo* over sulfuric acid and analyzed for chlorine.

0.1364 gm. salt gave 0.1080 gm. AgCl = 19.58 per cent Cl.
0.1348 " " " 0.1066 " AgCl = 19.56 " " Cl.
Calculated for stachydrin hydrochloride, Cl = 19.74 per cent.

Further evidence of the fact that the compound in question was stachydrin was afforded by the analyses and properties of the chloroaurate and the methyl ester chloroaurate. These were prepared from a sample of stachydrin hydrochloride obtained from different preparations.

Stachydrin Aurate.—0.6850 gm. of stachydrin hydrochloride

was dissolved in about 15 cc. of water, and a concentrated water solution of gold chloride added drop by drop until no further precipitation resulted. The precipitate was filtered off by suction on a hardened filter, washed with a little cold water, and then recrystallized from hot water. It was soluble with difficulty and upon concentration gathered as an oil in the bottom of the beaker. Cooling caused it to solidify at once partly in the form of plates of a rhombic habit. After 12 hours the aurate was filtered off, dried over phosphorus pentoxide *in vacuo* at room temperature, and then in an oven at 100° for 2 hours.

0.2836 gm. salt gave 0.1148 gm. Au = 40.48 per cent Au.
Calculated for stachydrin aurate, Au = 40.70 per cent.

In crystalline form the aurate corresponded well with the aurate described by Jahns.[10] It did not have a constant melting point, but started to decompose at about 185°. Yoshimura and Trier[11] found it to melt at 225–226°, but Jahns[10] obtained values varying from 190–210° with rapid heating, and states that the aurate has no definite melting point.

Stachydrin Methyl Ester Chloroaurate.—Approximately 1.8 gm. of stachydrin hydrochloride previously thoroughly dried, were dissolved in about 15 cc. of absolute methyl alcohol and dry hydrochloric acid gas was passed in for 1 hour. Slight warming resulted. The solution was transferred to a crystallizing dish and dried *in vacuo* over fused calcium chloride and soda lime. Though concentrated to a small volume the product failed to crystallize even after a few days. Cooling in the refrigerator did not induce crystallization. All that could be obtained was a viscid syrup of a faint yellow color. More drastic treatment was not used for fear of causing hydrolysis of the ester. A few drops of the ester freed from hydrochloric acid with silver oxide showed it to be decidedly alkaline in reaction. This is of interest in connection with stachydrin, as stachydrin hydrochloride freed from hydrochloric acid in a similar manner is neutral in reaction. Evidently in stachydrin the basicity of the nitrogen atom is intramolecularly neutralized by the carboxyl group. When the carboxyl group is taken care of by an ester linking, the basicity of the nitrogen atom again manifests itself. As the ester could not be purified as the hydrochloride, it was converted into the chloro-

aurate by adding to its aqueous solution a solution of gold chloride. Immediate precipitation resulted. From appearances it was more insoluble than stachydrin chloroaurate and apparently offered excellent opportunities for purification. The precipitate was filtered off by suction on a hardened filter, washed with a little cold water, and recrystallized from hot water. It did not dissolve readily, and on concentrating its aqueous solution it soon separated in oily droplets in the bottom of the beaker. On cooling, beautiful yellow plates crystallized out from the supernatant liquors. After 24 hours they were filtered off, dried over phosphorus pentoxide for 24 hours, and a portion was incinerated.

0.209 gm. gave 0.083 gm. Au = 39.71 per cent Au.

Calculated for stachydrin methyl ester chloroaurate, Au = 39.68 per cent.

The methyl ester chloroaurate was found to melt at 81–82° (uncorrected) which corresponds well with the value of 85° obtained by Schulze and Trier.[12] As aurates usually have no sharp melting the discrepancy is of no importance.

Optical Activity.—Stachydrin hydrochloride as obtained from most sources has been found optically inactive, but Yoshimura and Trier[11] have reported the isolation of *l*-stachydrin from *Galeopsis grandiflora* Lam. They found it to have a specific rotatory power of − 26.5°. Stachydrin hydrochloride from our preparation was found to be optically active, rotating the plane of polarized light to the left.

1.0224 gm. of the hydrochloride dissolved in 14.2 cc. of water in a 2 dm. tube gave a rotation of − 2.98°; $[\alpha]_D = -20.7°$. The low value may be due to partial racemization as no precautions were taken to prevent it, although it is possible that part of the stachydrin was originally present in the inactive form.

Quantitative Estimation.—350 gm. of alfalfa hay were extracted repeatedly in the course of 24 hours with a total of 4.8 liters of distilled water at the boiling temperature. The extract was precipitated with lead acetate, then freed from lead, precipitated with phosphotungstic acid, and treated as in the case of Preparation I using special precautions to minimize losses. The hydrochloride finally obtained was decolorized, evaporated to dryness, and dried at 95–100°. 2.787 gm. of a crystalline material

slightly brown in color were obtained. On boiling it with absolute alcohol, 0.415 gm. of a salt, which proved to be potassium chloride, was left as an insoluble residue. After filtration, the solution was concentrated to a small volume and cooled. A thick mass of crystals separated. After the addition of a little alcohol and ether they were removed on a suction filter, washed with a mixture of alcohol and ether, and dried at 100°. 0.876 gm. of pure white crystalline stachydrin hydrochloride was obtained. 0.1309 gm. on precipitation with silver nitrate gave 0.1056 gm. of silver chloride equivalent to 0.02612 gm. of chlorine or 19.95 per cent. Calculated for stachydrin hydrochloride, chlorine = 19.74 per cent.

The mother liquors on concentration failed to yield any further crops of crystals. On standing in a desiccator, however, they ultimately formed a solid mass. This has not yet been dissected, but undoubtedly contained some stachydrin hydrochloride.

The alfalfa hay from which this preparation was made was found on analysis to contain 3.88 per cent of nitrogen. In 350 gm. there would be 13.58 gm. of nitrogen. In 0.876 gm. of stachydrin hydrochloride containing 7.79 per cent of nitrogen there would be 0.0682 gm. or 0.5 per cent of the total nitrogen of the hay. This undoubtedly is to be looked upon as a minimum value so that it is seen that appreciable amounts of nitrogen in alfalfa occur in the form of stachydrin.

SUMMARY.

Stachydrin has been isolated as the hydrochloride from the phosphotungstic acid fraction of the water-soluble constituents of alfalfa hay. The picrate, chloroplatinate, aurate, methyl ester, and methyl ester chloroaurate were prepared and found characteristic. The optical activity though of the same sign was found slightly below the reported value which may have been due to racemization.

The presence of the base stachydrin in a feeding material like alfalfa shows the further necessity for caution in the use of values for histidine and lysine as obtained by the Van Slyke method and for lysine as obtained by the Kossel method when these values are obtained directly on the feeding material itself.

THE DETERMINATION OF CHOLESTEROL IN BLOOD SERUM.

By ADOLPH BERNHARD.

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(Received for publication, April 22, 1918.)

Many colorimetric methods have been described for the estimation of cholesterol in the blood. In a comparative study of the six general methods employed in the determination of cholesterol, Weston[1] found that Bloor's[2] method gave the most inconsistent results. Our experience with Bloor's method is in accordance with the findings of Weston.

In choosing a method for routine work, the method must give consistent results, and the one that meets this requirement is that introduced by Henes,[3] which utilizes the extraction method of Weston and Kent[4] and the colorimetric method of Grigaut.[5]

Henes' modification requires 5 days for completion, which is rather long for routine work. The revised method here presented can be completed in about 5 hours, and shortens the number of operations necessary to complete the determination. The results are almost identical with those obtained by the Henes method.

Revised Method.

The revised method obviates the necessity of extraction for 24 hours with alcohol and again for 24 hours with ether, since treatment with alcohol-ether mixture insures complete extraction of cholesterol almost immediately. By the use of the alcohol-ether mixture both steps are combined in one operation. The calcium hydroxide precipitate of cholesterol after saponification

[1] Weston, P. G., *J. Biol. Chem.*, 1916–17, xxviii, 383.

[2] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[3] Henes, E., Jr., *Proc. N. Y. Path. Soc.*, 1913, xiii, 155.

[4] Weston, P. G., and Kent, G. H., *J. Med. Research*, 1912, xxvi, 531.

[5] Grigaut, A., *Compt. rend. Soc. Biol.*, 1910, lxviii, 827.

is dried in an electric oven, instead of allowing it to dry in the air over night. An absolutely dry sediment is thus obtained. Treatment of this dry sediment with ether for half an hour is sufficient for complete extraction of the cholesterol, whereas Henes extracts the sediment with ether for 24 hours. The revised procedure as described is much more simple and rapid and is believed to be a considerable improvement over Henes' method.

Technique.—2 cc. of serum are pipetted into a 250 cc. Erlenmeyer flask of Pyrex glass, 25 cc. of hot 95 per cent alcohol added, and 100 cc. of a mixture of equal parts of 95 per cent alcohol and ether. Place on the water bath and bring to the boiling point; filter into a 250 cc. Erlenmeyer flask. Wash the precipitate once with about 25 cc. of warm 95 per cent alcohol. To the combined filtrate add about 1 gm. of stick sodium hydroxide, and boil on water bath for 2 hours. About one-fifth of the original volume remains at the end of this time. Add 100 cc. of a saturated solution of calcium hydroxide and collect the precipitate formed on a filter. Dry the filter and flask in an electric oven, not over 90°C., return the dry precipitate and filter paper to the flask, cover with 100 cc. of ether, and allow to extract for half an hour. Filter off the ether into a 150 cc. Pyrex extraction flask, wash the sediment with ether, then evaporate the ether on the water bath. To the dry sediment of cholesterol in the extraction flask add 5 cc. of chloroform. Transfer to a clean, dry test-tube, add 2 cc. of acetic anhydride (Merck) and exactly 4 drops of Baker's special sulfuric acid, mix thoroughly, and place in a dark place for exactly half an hour. At the end of this time compare in a Duboscq colorimeter with a standard solution of cholesterol in chloroform (5 cc. = 3 mg. of cholesterol) similarly treated as the unknown.

If 2 cc. of serum are used and standard is set at 10, the cholesterol per 100 cc. of blood may be obtained from the following formula:

$$\frac{10 \times 3}{R} \times 50 = \frac{1{,}500}{R}$$

10 = reading of standard, 3 = strength of standard, R = reading of unknown.

Table I gives the results of thirty-nine cholesterol determinations using both Henes' method and the short modification described above. The results are in close agreement with those obtained by the Henes method.

Attention is here called to the importance of the following in cholesterol determinations: (1) Absolutely clean and dry glassware is essential. (2) Pure reagents must be used; the acetic anhydride should be redistilled and is only of value when water-

TABLE I.

Case No.	Henes' modification.	Revised method.	Diagnosis.
	mg. per 100 cc.	*mg. per 100 cc.*	
S. P. 46	273	273	Cholelithiasis.
8,648	300	300	Nephritis with retention.
421	244	250	Diabetes.
447	375	375	Acute nephritis.
496	250	250	Bronchitis.
454	205	200	Lobar pneumonia.
386	188	188	Intestinal hemorrhage.
523	110	110	Pernicious anemia.
450	188	188	Pleurisy with effusion.
F. 1 T.	136	130	Cataract.
451	250	214	Diabetic gangrene.
643	210	205	Prostatic abscess.
504	250	250	Rheumatic endocarditis.
F. 2 623	142	136	Incomplete abortion.
662	70	87	Pulmonary tuberculosis.
547	100	96	Tuberculosis.
638	95	100	Pneumonia.
M. 3 K.	225	200	Diabetes.
815	165	180	Gastroptosis, achylia gastrica.
893	176	160	Lobar pneumonia.
799	150	150	Pneumonia.
109	250	240	Neoplasm of lung.
814	350	370	Chronic nephritis.
Hem.	150	135	Cardiac decompensation.
M. 1 Ho.	115	120	" " angina pectoris.
F.	250	240	Arthritis.
M. 3 M.	167	176	Cholelithiasis.
M. 1 He.	231	231	Diabetes.
F. 1 L.	333	333	"
F. 1 F.	150	150	Lues.
F. 1 W.	150	163	Secondary anemia (pregnancy).
F. 1 A.	125	120	Rheumatism, mild diabetes.
Cur.	231	250	Chronic nephritis.
953	215	228	Thromboangiitis obliterans.
M. 1 K.	290	293	General anasarca, nephritis.
F. 1 K.	260	273	Hematemesis.
M. 2	150	150	Acute nephritis.
Mat.	231	231	Thromboangiitis obliterans.
Bash.	186	188	" "

clear. The sulfuric acid must be free from water. Baker's special sulfuric acid is recommended. The chloroform should be redistilled before using. The calcium hydroxide solution should be made up fresh about every 3 weeks. The standard solution of cholesterol should be kept in a dark colored, glass-stoppered bottle, and will keep indefinitely.

SUMMARY.

A revised procedure for the determination of cholesterol in the blood is described. It is a modification of Henes' adaption of the extraction method of Weston and Kent and the colorimetric method of Grigaut. The determination can be completed in 5 hours and gives results in close agreement with those obtained by Henes' adaption.

THE CHOICE BETWEEN ADEQUATE AND INADEQUATE DIETS, AS MADE BY RATS.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Edna L. Ferry and Alfred J. Wakeman.

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(Received for publication, May 3, 1918.)

The success with which animals living in their natural environment nourish themselves must depend upon the exercise of choices in which instinct and appetite play a significant part. When such individuals are transferred to conditions of domestication their freedom of choice, particularly in the case of captive or farm animals, is restricted. Their diet now becomes a problem for the husbandman who follows in part his observations on the actual feeding habits of the species and in part the dictates of special trials or experimental observations on the species in question. Many infants and children are likewise dependent upon a dietary regimen controlled or modified independently of their natural preferences.

Man generally chooses his own food; but the selection is limited by a number of circumstantial factors which have led to the formation of so called dietetic habits. In discussing the origin of modern so called feeding standards, Armsby[1] says:

"As soon as live stock husbandry emerged from the pastoral stage and man began to store up forage for the winter or to utilize the products of his cultivated land for feeding his domestic animals, the question of the relative values of the different feeding-stuffs necessarily arose. As agriculture has gradually become more intensive and as the variety of nat-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Armsby, H. P., The nutrition of farm animals, New York, 1917, 591.

ural materials and of technical by-products available has increased, the question has grown in importance, the traditions of practice based on the experience of earlier investigations have been recognized to be insufficient guides, and much effort has been put forth to replace these traditions by exact knowledge."

Progress in the study of nutrition has tended to show a far greater complexity in the nutritive needs of the organism than was appreciated or even suspected until recently. One by one the energy factor, the quantity and suitability of the proteins, the inorganic aspects, the roughage or ballast, and the vitamine features of the diet have come into prominence and demanded recognition in the construction of an adequate dietary. In the midst of this tendency in somewhat academic directions one may inquire whether the lessons derived from the observations of the natural feeding habits of both man and the domestic animals are not being unduly neglected. In other words, are there no lessons to be learned from the exercise of the feeding instincts in the voluntary selection of foods?

It must be admitted at once that *appetite* is by no means an unerring guide, as the perversions in the use of tea, coffee, alcohol, tobacco, etc., show. Nevertheless the biological evidence of the survival and well-being of innumerable species testifies to the success of their *instinctive* dietary habits. How unwise artificially established "standards of feeding" may be has well been illustrated by the experiments of the Wisconsin Experiment Station on supposedly "balanced" rations derived from single plant sources.[2]

Evvard[3] has lately emphasized anew the importance for practical husbandry of taking account of the feeding habits of animals. He has instituted observations on the growth of swine which were permitted a *freedom of choice* among the following variety of food products: whole corn grain, meat meal (containing 60 per cent of protein), whole oats, linseed oil meal, wheat middlings, charcoal, limestone, salt, and water. The results obtained with pigs fed according to this "free choice" system were surprising in that the animals provided with nutrients in this way made remarkably

[2] Hart, E. B., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Station Bull. 287*, 1918.

[3] Evvard, J. M., *Proc. Iowa Acad. Sc.*, 1915, xxii, 375.

rapid growth, one of them being, indeed, the largest pig for its age ever raised at that Experiment Station. Moreover, the pigs which received "sodium chloride *ad libitum* made more rapid growth (increase in weight) with less requirement of feed per unit increase than when no salt was allowed, or when it was mixed in variable quantities with a basal ration of corn grain and linseed oil meal (whole flax grain minus most of the oil)." Indeed, comparative trials showed that either smaller or larger quantities of salt led to less satisfactory growth than the optimum selected by the animals exercising a freedom of choice.

In commenting upon the outcome of the experiment Evvard says:

> "One dominant reason for the sometimes ineffective results secured in feeding according to ordinary standards is the difficulty of knowing the resultant of the many, specific units, as many as a hundred, which in all probability are comprised in an ordinary feeding-stuff such as maize grain, or meat meal, or oats, or oil-extracted flax grain residue. The suitability of different individual feeds for the animals in question has been determined largely from a careful observation of results secured in practical animal husbandry. This general knowledge of practical feeding-stuffs and their effects is of great advantage in supplementing the best feeding standards. At best the "best" standard available today is but a crude approximation of actual requirements. When the appetite is given full control of what shall be eaten it is surprising to note how the pigs naturally select the specific feeds which swine herdsmen have long since approved as of the best, and what is equally surprising the pigs show marked avoidance of those feeds usually considered as ill-adapted to swine."

Observations which have been accumulated in our experimental work during recent years have likewise demonstrated the somewhat surprising ability of rats to make selections between two food mixtures of somewhat similar physical character, one of which may be described briefly as nutritively inferior in contrast with the other that was more adequate for nutrition in growth. Although the rats usually ate some of each kind of food offered, as a rule they ate more of the adequate food than of the inferior one.

The effect of this "free choice" system of feeding the rats is shown in the appended curves of growth. In the graphic representations of the food intake at the bottom of each chart the superior food is represented by the shaded areas, and the inferior

food by the unshaded ones, the sum of these areas representing the total food intake.

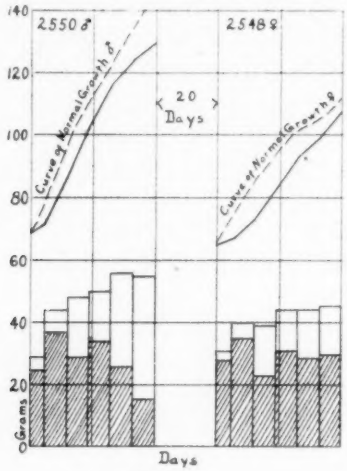

CHART I.

Chart I shows the outcome of a choice between two foods alike in all respects except the single protein incorporated in each mixture. The composition of the two food mixtures was as follows:

	Superior mixture. *per cent*	Inferior mixture. *per cent*
Lactalbumin	18	
Zein		18
Protein-free milk	28	28
Starch	28	27
Butter fat	18	18
Lard	8	9

In previous communications[4] we have shown that this zein food mixture promptly leads to nutritive decline owing to the deficiency of the protein in lysine and tryptophane groups. When supplemented by these amino-acids or by a sufficient amount of a suitable protein, however, the previously inadequate diet can be rendered entirely adequate for nutrition in growth. In the pres-

[4] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington Publication 156*, pt. ii, 1912; *Z. physiol. Chem.*, 1912, lxxx, p. 307; *J. Biol. Chem.*, 1914, xvii, 325; xviii, 1; 1915, xx, 351; 1916, xxv, 1.

ent experiments it will be noticed that the Rats 2548 and 2550 actually ate some of each food mixture, the outcome of the choice resulting in fairly satisfactory growth. The success of the mixed intake did not depend upon the ingestion of an inordinate total amount of food, so that the inferior mixture was superimposed upon a sufficient quantity of the superior one; for the total intakes recorded approximate those which we have found to be commonly selected by animals growing upon adequate rations of the same type consumed *ad libitum*.[5] It is not easy to imagine what factor impelled so successful a choice of diet.

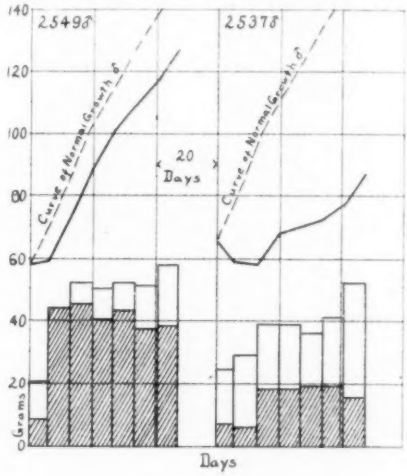

CHART II.

An equally difficult choice is indicated in the results presented in Chart II indicating the outcome when two foods precisely alike except in their content of the same protein (casein) were offered. The food mixtures had the following composition:

	Superior mixture. *per cent*	Inferior mixture. *per cent*
Casein	18	4.5
Protein-free milk	28	28.0
Starch	29	31.5
Sucrose		10.0
Butter fat	18	18.0
Lard	7	8.0

[5] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351.

Rat 2549 accomplished the choice successfully in that a preponderating intake of the food richer in protein made growth possible. The result with Rat 2537 may perhaps be looked upon as a failure to make a choice suitable from the nutritive standpoint. It is not unlikely, however, that if the experiment had been continued, a successful selection would ultimately have

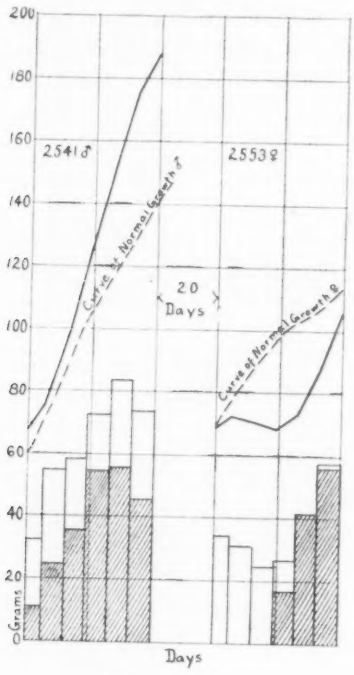

CHART III.

ensued. Such an outcome is shown in Chart III, Rat 2553, in trials in which the difference between the two foods offered consisted in their content of water-soluble vitamine derived from milk. This animal reversed its food preference at the end of 3 weeks and succeeded in growing. Its companion, Rat 2541, effected a highly suitable selection in much shorter time. The point to be emphasized however is that ultimately both animals made good choices.

The composition of the foods was as follows:

	Superior mixture. *per cent*	Inferior mixture. *per cent*
Casein	18	18
Protein-free milk	28	
Artificial protein-free milk IV*		29
Starch	29	28
Butter fat	18	18
Lard	7	7

* The preparation of this mixture is described in *J. Biol. Chem.*, 1913, xv, 317.

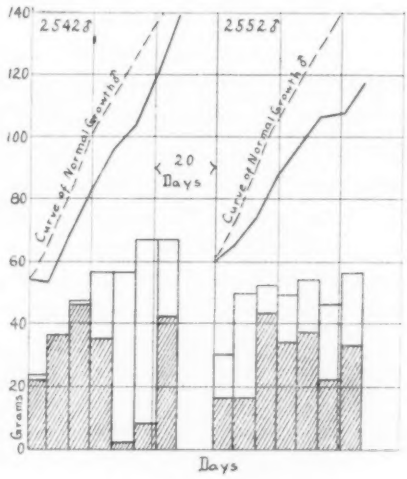

Chart IV.

Chart IV represents the results of a choice between two food mixtures, neither of which has a sufficient concentration of protein to serve ideally for the purposes of growth. With this 9 per cent lactalbumin food fairly good growth may sometimes be attained;[5] this is not the case with the comparable 9 per cent casein food unless enough is eaten to supply the required quantity of the poorer protein.

The composition of the mixtures was as follows:

	Superior mixture. *per cent*	Inferior mixture. *per cent*
Lactalbumin	9	
Casein		9
Protein-free milk	28	28
Starch	29	29
Sucrose	9	9
Butter fat	18	18
Lard	7	7

It will be observed that despite the considerable variations in the choice of foods from week to week the resultant tendency was toward better growth than the inferior food mixture would permit. During the 5th and 6th weeks Rat 2542 ate the inferior food almost exclusively, but it will be noted that it made good the deficiency in the proportion of the inferior protein in this food by a greater intake, thereby securing a relatively larger absolute amount of protein.

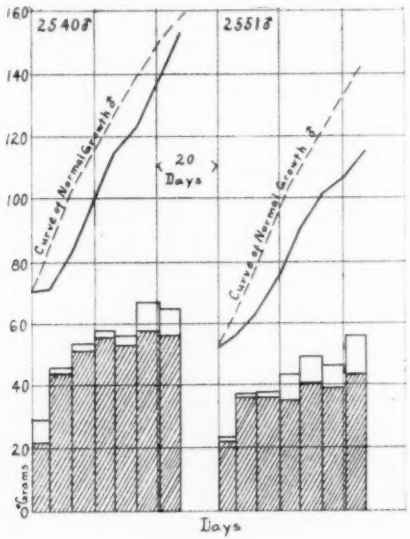

CHART V.

Finally, Chart V shows the choice between an adequate food and a somewhat comparable mixture entirely inadequate because it

was almost devoid of protein. The composition of the two foods was as follows:

	Superior mixture. *per cent*	Inferior mixture. *per cent*
Casein	18	
Protein-free milk	28	28
Starch	29	27
Sucrose		11
Lactose		10
Butter fat	18	18
Lard	7	6

Presumably the inferior food was not unpalatable; for it contained a not insignificant proportion of cane sugar which rats usually like to eat. Nevertheless the choices were such as to facilitate growth.

In surveying the comparatively meager data here presented we are unwilling to draw any far reaching conclusions. It seems to be more than a coincidence, however, that the choices which the rats have made correspond so well with what our previous experience with these foods had already demonstrated to be preferable from the standpoint of growth. Although in these few experiments the growth was not the maximum that might have been expected, nevertheless nine out of ten rats soon ate such proportions of the two foods offered in each case as to enable them to grow at a nearly normal rate. It is difficult to imagine what the impulse is which led the rats to make these selections; for numerous experiments which we have made prove that the inferior foods offered to those shown in Charts II, III, and IV were entirely adequate to maintain them in good condition for very long periods. It is therefore interesting to have this evidence that the desire of a young animal for food is something more than the mere satisfaction of its calorific needs. The demand made by the growth impulse must also be met by a food of the proper chemical constitution.

THE AMINO-ACIDS OF MATURE HUMAN PLACENTA.

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(Received for publication, April 8, 1918.)

There has always been a keen interest in the chemical nature of the placenta. The exact nature of the rôle it plays in the nutrition of the fetus, its importance in some of the theories of eclampsia, and latterly, that its hydrolysis by normal and pregnant sera has been made a touchstone for the Abderhalden theories of specific defensive ferments, combine to render its chemical examination one of the highest interest.

The earlier work on the grosser chemical examination has been ably presented in this *Journal* by Koelker and Slemons[1] and its repetition here serves no useful purpose. The following points, however, might be noted. (1) About one-half of the fresh normal placenta consists of blood. (2) 82 to 85 per cent is water. (3) The ash varies from 0.87 per cent to 1.15 per cent. (4) The fat in unwashed placenta is 0.846 per cent, and in washed placenta 0.535 per cent. (5) The carbohydrate is glycogen (0.49 to 0.58 per cent), which disappears rapidly after delivery, sinking to about half value in 20 minutes. (6) The main part of the solids is protein. The nitrogen in dry, ash-free, and fat-free placenta, according to Koelker and Slemons, is 14.9 per cent. (7) Wells and Corper[2] give about 2.1 per cent of the total nitrogen as purine nitrogen.

The amino-acids of the human placenta protein have been examined by Koelker and Slemons. They used the Fischer and the Kossel methods of separating the amino-acids, and their results did not call for any striking comment. Their most noteworthy finding was the apparent absence of alanine.

We felt, however, that a reexamination of the subject would be of value, using the Van Slyke method for the distribution of the protein nitrogen.

[1] Koelker, A. H., and Slemons, J. M., *J. Biol. Chem.*, 1911, ix, 471.

[2] Wells, H. G., and Corper, H. J., *J. Biol. Chem.*, 1909, vi, 469.

EXPERIMENTAL.

Preparation of the Placenta.

The placentas used in this work were normal full term placentas obtained from the Montreal Maternity Hospital.[3] Immediately after delivery the placentas were placed in a jar of physiological saline, covered with a layer of toluene, and the jar was sealed. They were brought to the laboratory within a few hours and were always in good condition. At the laboratory the placentas were treated somewhat after the manner of Abderhalden,[4] in preparing a substrate for the Abderhalden pregnancy test. The umbilical cord and adhering membranes were completely removed. At the same time, as many as possible of the larger blood vessels were excised, the organ being torn into small pieces in the process. These were rinsed several times in cold water and wrung out in cheese-cloth. They were next passed through a mincing machine, and the finely divided, pulpy mass was washed with water in tall glass cylinders, to the bottom of which passed a glass tube connected with the water tap. A gentle stream of water was thus passed through the cylinders, for a period of from 6 to 10 hours, insuring a thorough removal of the blood. A piece of cheese-cloth tied over the top of the cylinder acted as a filter, preventing the loss of finer pieces of placenta. Even with a thorough washing of this kind, some placentas still retained a trace of pink color. These were rejected. We found, also, that sometimes even those placentas which after washing appeared perfectly white and bloodless, on standing in saline solution contributed a pinkish color to the fluid.

The mass of washed placenta was then coagulated by boiling in water rendered faintly acid by acetic acid. Three separate boilings were made, using fresh portions of acidified water, the placentas being placed in cheese-cloth and squeezed dry between each operation. The length of time of each boiling was about 10 minutes. After the final boiling, the excess water was thoroughly removed by a hand press and the material spread out in a thin layer on coarse filter paper, and left to dry over night at a temperature of about 25°C. The dried placenta was then ground in a hand mill to a fine powder, the coarser particles being removed by a fine wire sieve and being again passed through the mill. In this way a fine uniform powder was obtained of a yellowish color, and with a slight characteristic odor. All the placentas were full term, normal specimens. No calcareous placentas were used. The final material was preserved in stoppered bottles.

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[3] We wish to acknowledge our indebtedness to the Hospital authorities, whose kindness and cooperation have contributed materially to the success of the research.

[4] Abderhalden, E., Handb. biochem. Arbeitsmethoden, Berlin and Vienna, 1912, vi, 226.

By weight this powder only represented about 3 per cent of the weight of the fresh organ, whereas the product used by Koelker and Slemons represented 7 per cent of the weight of the fresh organ. Our treatment, however, had been a much more vigorous one, and the difference may be readily accounted for by mechanical losses as well as by the solution of salts and albumin. An average sample of eleven placentas was thus prepared.

Nitrogen Content of the Placenta Preparation.

Koelker and Slemons[1] give the percentage of nitrogen in their dry ash-free and fat-free preparation of placenta as 14.9. Our own results agree very closely, although we had submitted the organs to a much more vigorous treatment.

Water.—The water remaining in the air-dried preparation was determined by the loss of weight when dried to constant weight at 100°. A mean of five determinations gave 9.50 per cent.

Ash.—Determined by ignition to constant weight in a platinum dish at low red heat. Mean of three determinations, 5.07 per cent.

Fat.—The fat was determined by Soxhlet extraction with ether on the oven-dried (100°) material. Two determinations gave 1.28 per cent. Corrected to air-dried material = 1.15 per cent.

Nitrogen.—Determined by Kjeldahl on air-dried material. Mean of seven determinations, 12.44 per cent. Corrected to dry, ash-free, fat-free material = 14.7 per cent.

Methods of Analysis.

The method used was that of Van Slyke[5] for the partition of the protein nitrogen, using the amyl alcohol extraction method for the removal of phosphotungstic acid, and the micro-apparatus for the determination of amino-acid nitrogen.

Time of Complete Hydrolysis.

We first determined the time of complete hydrolysis of the placentas by 20 per cent HCl, as recommended by Van Slyke. 3 gm. of placenta were boiled under a reflux condenser and a rough determination of amino-acid nitrogen was made at the end of 8

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15; 1912, xii, 295; 1915, xxii, 281.

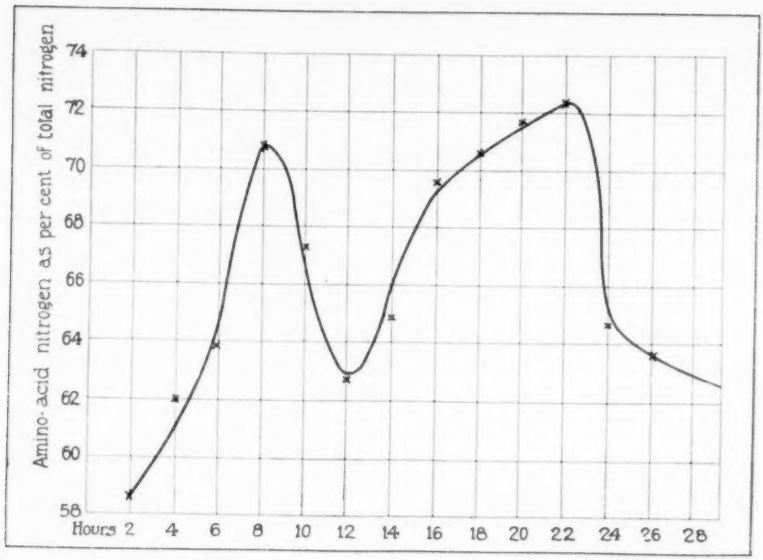
Amino-acid nitrogen as per cent of total nitrogen
74
72
70
68
66
64
62
60
58
Hours 2 4 6 8 10 12 14 16 18 20 22 24 26 28

FIG. 1.

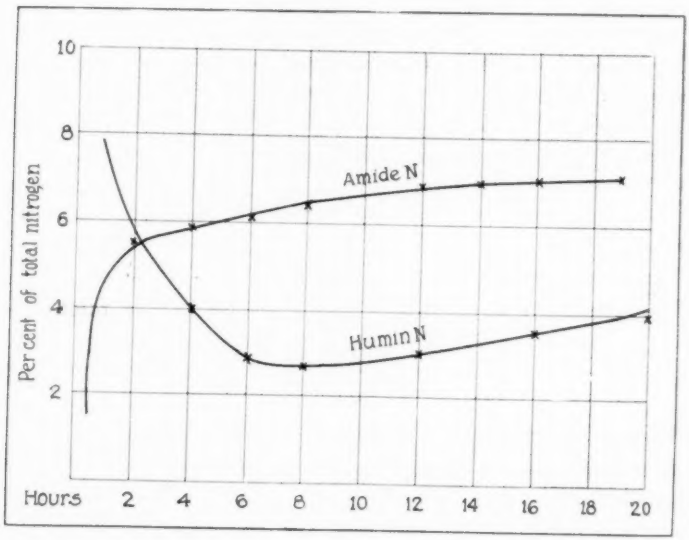
Per cent of total nitrogen
10
8
6
4
2
Amide N
Humin N
Hours 2 4 6 8 10 12 14 16 18 20

FIG. 2.

hours and again at the end of 12 hours. The latter value was found to be considerably lower than the first. As we were evidently dealing with a very easily hydrolyzed protein, we carried out a hydrolysis under the same conditions, removing a sample of 0.5 cc. every hour for analysis, for a period of 24 hours, and then occasionally till the end of 57 hours. The ammonia was not removed. The results are shown in Fig. 1 and revealed that there were two maxima, one at about 8 hours and a second at about 22 hours. These results were confirmed by a second hydrolysis, re-

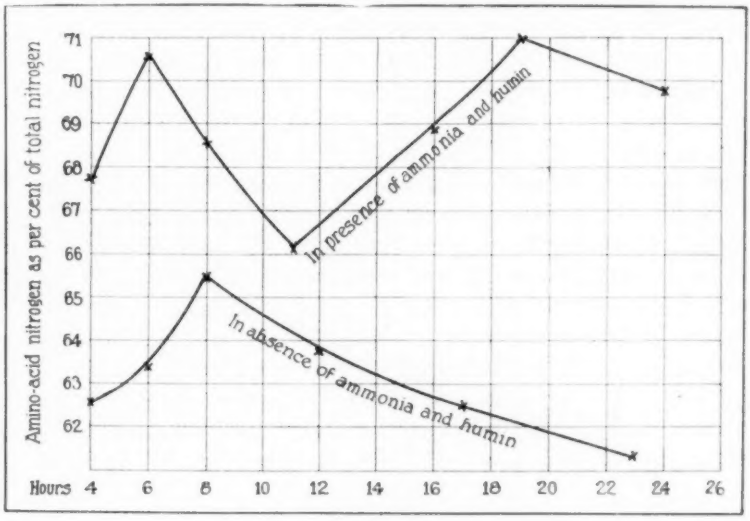

FIG. 3.

moving samples for analysis every 2 hours, though the second maximum in this case did not quite coincide with the previous determination. The natural interpretation to place upon these results is that we are dealing with two proteins in our placental preparation, one hydrolyzed with great ease and a second more resistant to hydrolysis. As the result was of decided novelty and opened up the possibility of a new application of the Van Slyke amino-acid nitrogen determination on the question of the chemical identity and chemical unity of insoluble proteins, we decided to investigate the matter further. A series of hydrolyses were carried out with 1 gm. of placenta powder and 20 cc. of 20

per cent HCl for periods of 2, 4, 6, 8, 12, 16, and 24 hours. At the end of the stated period a sample was withdrawn and the ammonia determined as in the determination of ammonia in urine by the Denis[6] micro-aeration method. We used this method to act as a control on the main determination, and found that it gave excellent agreement. A determination of the amino-acid nitrogen was also made. The hydrolysis products were cooled, transferred to small vacuum distillation flasks, and ammonia was determined, following the procedure of Van Slyke. The humin was also determined in each, and then again the amino-acid nitrogen, freed this time from the influence of ammonia and humin. We felt that no conclusions could be drawn on the existence of two proteins until such a procedure was carried out. The results are shown in Figs. 2 and 3 and fully justify the caution we exercised. There is no evidence of the existence of the second protein, the point of maximum hydrolysis being at about 8 hours. This indicates an extraordinarily easily hydrolyzed protein. The ammonia is produced practically entirely in the first 2 hours, and then slowly and continuously increases, facts which are in agreement with those of other observers. The humin, large at first on account of unhydrolyzed protein, becomes a minimum at about 8 hours and then slowly increases. The point of maximum amino-acid production was thus fixed at about 8 hours, and we can offer no adequate explanation of the two maxima obtained in our previous experiments.

Nitrogen Distribution in Placenta.

All analyses were carried out in duplicate, and previous to the determinations reported here, we made ourselves familiar with the technique by a preliminary series of experiments.

The figures in Table I show the nitrogen distribution in placenta, being given as percentages of the total nitrogen, corrected for the solubility of the phosphotungstates, and the placentas representing an average sample of eleven normal organs.

We also studied the individual variations by examining five placentas in a similar manner. The placentas were separated, washed, coagulated, air-dried, and ground exactly as the previous average sample. The nitrogen in each air-dried specimen was

[6] Denis, W., *J. Biol. Chem.*, 1910–11, viii, 427.

first determined, and was found to be remarkably constant. This is no doubt due to the conditions of preparation being the same, as all five specimens were prepared practically simultaneously.

Placenta	A	B	C	D	E
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Nitrogen	13.54	13.25	13.52	13.68	13.54
	13.50	13.22	13.52	13.54	13.50

The nitrogen distribution is shown in Table II, the figures as before being given as percentages of the total nitrogen, and corrected for the solubility of the phosphotungstates. The general findings corroborate those of the average sample.

TABLE I.

Nitrogen distribution.	I	II
	per cent	*per cent*
Amide	6.34	6.46
Humin	3.20	3.15
Arginine	24.08	23.92
Histidine	2.32	2.55
Lysine	7.34	7.28
Cystine	1.47	1.39
Total diamino	35.21	35.14
Monoamino	51.55	51.90
Non-amino	3.47	3.28
Total recovered	99.89	99.79

DISCUSSION.

The most striking result is the high value obtained for arginine. This is seen both in the average sample and the individual specimens of placenta, and is not due to any analytical error. We have confirmed the high figure by obtaining a similar value by hydrolysis under totally different conditions (p. 40). This high arginine content of placenta, made according to the Van Slyke distribution method, places this organ in a category quite apart from that of all other human organs.

In Table III is given the analysis of various human organs, carried out by Drummond.[7] The preparation of the organs was similar to ours of placenta, and the analytical methods were

[7] Drummond, J. C., *Biochem. J.*, 1916, x, 473.

TABLE II.

Placenta	A			B			C			D			E		
Nitrogen distribution.*	I	II	Average.	I	II	Average.	I	II	Average.	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Amide	6.36	6.33	6.34	6.57	6.48	6.53	6.65	6.54	6.59	6.40	6.44	6.42	6.26	6.22	6.24
Humin	2.90	2.97	2.94	2.93	2.97	2.95	2.90	2.92	2.91	2.95	2.99	2.97	2.85	2.90	2.88
Arginine	22.74	22.95	22.85	22.92	23.07	22.99	22.07	21.85	21.96	24.16	24.02	24.09	19.51	19.30	19.40
Histidine	1.08	0.95	1.02	2.55	2.37	2.46	4.32	3.58	3.95	4.54	4.30	4.42	4.30	4.61	4.45
Lysine	8.33	8.32	8.33	6.45	6.52	6.49	5.44	5.96	5.70	6.51	6.71	6.61	8.89	8.82	8.86
Cystine	1.29	1.26	1.28	1.58	1.54	1.56	1.54	1.51	1.53	1.14	1.11	1.13	1.20	—	1.20
Total diamino			33.48			33.43			33.14			36.25			33.91
Monoamino	51.63	51.51	51.57	52.36	52.70	52.53	49.67	50.41	50.04	50.61	50.34	50.48	50.40	50.70	50.55
Non-amino	6.79	6.77	6.78	4.51	3.72	4.12	8.06	6.99	7.53	5.65	5.94	5.80	7.78	7.21	7.50
Total recovered	101.12	101.06	101.09	99.86	99.24	99.69	100.75	99.76	100.21	101.82	101.75	101.78	101.18	100.96	101.08

those of Van Slyke, so that the results become quite comparable although the figures are not corrected for the solubility of the phosphotungstates.

The various organs analyzed are representative of nearly all types of cells and we included the lung tissue of a chicken to make the representation more complete. They do not show any very striking variations; in fact, except for the histidine content, they, are remarkably uniform in composition. The average figures,

TABLE III.

Organ.	Nitrogen distribution.								
	Amide.	Humin.	Arginine.	Histidine.	Lysine.	Cystine.	Total diamino.	Monoamino.	Non-amino.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Pancreas	5.52	4.50	11.30	8.82	10.91	0.97	32.00	53.27	3.63
Kidney	5.05	3.24	11.95	5.89	7.00	1.01	25.85	59.30	5.10
"	6.05	3.57	11.45	8.60	9.25	1.10	30.40	52.80	5.82
Spleen	5.18	3.82	12.50	13.50	11.00	1.10	38.10	47.62	4.88
Liver	6.12	3.32	10.50	8.20	11.76	1.34	31.80	53.85	4.35
Breast (slightly fibrotic)	5.50	2.50	11.10	4.66	12.47	—	27.23	57.17	6.40
Breast (slightly fibrotic)	5.10	2.85	10.02	2.81	13.38	—	26.21	56.20	6.00
Skeletal muscle	6.18	2.29	11.05	2.72	13.60	0.80	28.17	58.90	4.41
" "	6.42	2.30	11.30	5.79	12.71	0.90	30.70	57.00	4.20
Cardiac muscle	5.67	1.55	9.38	10.20	12.50	0.82	32.90	52.95	5.90
" "	6.35	3.60	10.00	10.00	9.25	0.80	30.05	53.20	5.00
Lung (chicken)	7.02	4.54	11.25	7.22	11.11	1.32	30.90	57.20	7.40
" "	6.58	3.82	12.20	8.42	10.98	1.40	33.00	50.70	6.70

also given by Drummond and compared with our figures for placenta, appear in Table IV.

On comparing the table of average values with the individual values it will be seen that the variation of the figures about the mean for the human organs is not very great, so that the average figures can be taken as representative. The exception is the value given to histidine. The same is true of our figures for normal human placenta. Our biggest percentage variation occurs in the figures for histidine. This is not surprising when we con-

sider that the histidine value is obtained indirectly from the sum of the arginine, lysine, and cystine values, and that small errors in these determinations might well become cumulative in either direction and so lead easily to an abnormally high or low histidine figure. So that, although we find a big difference between the average histidine content of normal human tissues as contrasted with that of normal human placenta, we do not feel inclined to lay any great stress on this at present. We would merely point out that although the placenta possesses a low histidine content as compared with other organs, it cannot be regarded as being deficient in that particular amino-acid.

TABLE IV.

Nitrogen distribution.	Normal human tissues. (Drummond.)			Normal human placenta. (Harding and Fort.)		
	Maximum.	Minimum.	Average.	Maximum.	Minimum.	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Amide	6.42	5.05	5.82	6.59	6.24	6.42
Humin	4.50	1.55	3.09	2.97	2.88	2.97
Arginine	12.50	9.38	11.13	24.09	19.40	22.54
Histidine	13.50	2.72	7.89	4.45	1.02	3.12
Lysine	13.38	7.00	9.83	8.86	5.70	7.22
Cystine	1.34	0.80	0.93	1.56	1.13	1.36
Total diamino	38.10	25.85	30.71	36.25	33.14	34.24
Monoamino	59.30	47.62	55.88	52.53	50.04	51.15
Non-amino	6.40	3.63	4.94	7.53	4.12	5.85

The same is true of its lysine content. Our average figure is 7.22 as compared with 9.83 for other organs. And although our maximum figure is less than the minimum figure, with one exception, given by Drummond for the other organs, we cannot consider the difference as any striking departure from the normal average. We have not yet reached that stage in our knowledge when small differences of that kind are intelligible.

With the arginine content, however, a very different picture is presented. With the normal human organs, the variation of this figure is very small. In fact the figures are surprisingly uniform, and, if our own experience with placenta may be taken as a criterion, might almost express the individual variation of a particular organ instead of a number of different organs. The difference between the two sets of figures is so constant and so

striking as to be above any accidental combination of circumstances, and leads us to the conclusion that in the placenta we are dealing with a type of organ fundamentally different from others. And with difference in chemical composition so striking, one cannot help but correlate difference in function. The human organs analyzed by Drummond include various types of secretory and excretory cells,—breasts, liver, pancreas, spleen, lungs, and kidney, as well as muscle, both plain and voluntary,—and in view of these facts one cannot easily ascribe to the placenta in its relation to the nutrition of the fetus a merely passive rôle. Thus the chemical composition of the protein of the placenta falls in line with a growing mass of evidence, and a now almost universally accepted belief, that it is an active organ of nutrition for the fetus. In this connection it is interesting to note that the breast, itself an active organ concerned with nutrition and an organ capable of elaborating special proteins and a special carbohydrate, does not differ markedly in composition from the remainder of the human body, though Drummond's figures probably are for the inactive organ. In its active state the breast may show quite a different picture. In attempting to interpret the high arginine values obtained, one must not lose sight of the fact that the placenta is one of the most rapidly growing organs in the female body, and that this, in conjunction with its origin, might account for the results. Kocher,[8] indeed, attempted to show that the growth of tumors was dependent on lysine, and claimed big differences between the rapidly growing tumors and the slower growing normal organs in their content of that amino-acid. Unfortunately it has been shown that Kocher's comparisons were based on inaccurate analyses of the normal organs,and that the rapidly growing tumors are very similar in their amino-acid make-up to the remainder of the animal body.

The high figure, however, which we have obtained for arginine comes into direct conflict with the amount obtained by Koelker and Slemons, who actually isolated the arginine. The figures of Table V are taken from their paper.

The last column shows the calculation of the various fractions of nitrogen expressed as percentages of total nitrogen from Koelker and Slemons' own figures, those in parentheses being ours

[8] Kocher, R. A., *J. Biol. Chem.*, 1915, xxii, 295.

for the sake of comparison. The figure for ammonia is approximately correct, whereas the lysine, arginine, and histidine figures are in all cases enormously lower than those obtained by ourselves, though the relative order is the same. To assure ourselves still further on the correctness of our results, we carried out a hydrolysis of placenta by sulfuric acid at a temperature of 105° and for a period of 24 hours; *i.e.*, a hydrolysis under the conditions prescribed for the determination of the hexone bases by the Kossel and Kutscher method. The sulfuric acid was removed, ammonia and humin were removed, and the resulting solution was analyzed for arginine by boiling an aliquot part with 20 per cent NaOH, following the directions of Plimmer.[9] The results obtained were always in substantial agreement with our previous figures, and as the placenta preparation used in these

TABLE V.

Amino-acid.	Amount in 500 gm. of placenta.	N per 100 gm. protein.	N as percentage of total N.
	gm.		
Ammonia	—	1.054	7.07 (6.42)
Lysine	16.00	0.664	4.46 (7.22)
Arginine	20.00	1.394	9.63 (22.50)
Histidine	1.10	0.064	0.42 (3.12)

determinations represented an entirely different average sample of over twenty organs, we felt confidence in our results. Still in view of the serious discrepancies between our results and those of Koelker and Slemons, we undertook to carry out a fractionation of the hexone bases according to the method of Kossel and Kutscher, and at the same time control the fractionation by determining at each step the arginine content of the precipitates and filtrates by the alkali hydrolysis method of Van Slyke or Plimmer. In this way any chemical separation of arginine from a second substance simulating arginine in its behavior towards alkali would at once be noticed. No such separation, however, occurred. With each fractionation we noted losses in arginine, due doubtless in part to imperfections in the method and to imperfections in our own technique. We did succeed, however, in isolating as an

[9] Plimmer, R. H. A., *Biochem. J.*, 1916, x, 115.

impure picrolonate an amount of arginine representing about 15 per cent of the total nitrogen. We feel, therefore, that our figure for arginine is a correct one.

Many interesting questions arise as to the origin and meaning of this storage of arginine in the placenta. The recent work of Ackroyd and Hopkins[10] on the connection between arginine (with histidine) and purine metabolism at once leads to the hypothesis that the rapidly growing fetus uses the arginine as the source of the purine part of its new nuclear material. The question, too, of postpartum creatinuria has so far received no satisfactory solution. With the expulsion of the placenta at birth, does the mechanism, which allowed of the storage of arginine, continue for some time to accumulate it in the muscles of the mother and produce a temporary creatinuria until conditions readjust themselves to the normal?

SUMMARY.

A determination of the amino-acids in mature human placenta has been made according to the nitrogen distribution method of Van Slyke.

The distinguishing feature of the placenta protein is a high arginine content.

This is double the arginine content found in other human organs (breast, liver, heart, kidney, muscle).

Corresponding to this difference, there must be ascribed to the placenta a function differing from that of other organs.

The placenta from a chemical standpoint cannot be regarded entirely as a passive organ.

[10] Ackroyd, H., and Hopkins, G. H., *Biochem. J.*, 1916, x, 551.

THE RATE OF COLOR PRODUCTION IN ALKALINE SOLUTIONS OF DEXTROSE AND PICRATE.*

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(Received for publication, April 15, 1918.)

When a mixture of dextrose and sodium picrate dissolved in water is heated in the presence of an alkali, a red-brown color is produced which exactly resembles the color of sodium picramate. This fact has been made the basis for the quantitative estimation of small amounts of dextrose and has been extensively used in determining the concentration of dextrose in blood. The method rests on the assumption that there is a direct proportion between the amount of color produced and the amount of dextrose present in the mixture.

We have found that this assumption is incorrect. Under most conditions there is a considerable deviation from a direct relationship, and we have failed to find any under which the agreement is absolute, though there was one particular complex of factors with which an almost direct proportion between color production and dextrose concentration was obtained.

The main technical difficulty lay in devising means whereby different temperature levels could be attained and kept constant for varying lengths of time. We finally succeeded in constructing an instrument by means of which all degrees of temperature from 80–130°C. could be uniformly maintained over any desired period of time. A small autoclave, such as is used by housewives for sterilizing fruits and vegetables for canning, was provided with an electrical source of heat and with an electrical thermostat. The pressure guage was inaccurate so a thermometer was added. An inner compartment of wire-lined asbestos was

* A summary of these results was published in *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 79.

also constructed in order to protect the solutions from inequalities of temperature arising from the circulation of water through the base of the autoclave (see Fig. 1).

Dextrose concentrations of 0.5, 0.1, and 0.05 per cent were made in saturated picric acid solution, corresponding to hyperglycemic, normal, and hypoglycemic blood specimens. Since blood is commonly diluted to two and a half times its volume with picric acid solution for the removal of protein, our solutions were

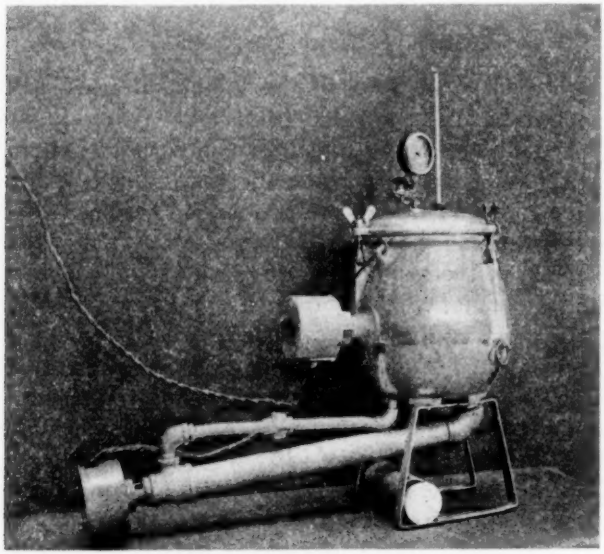

FIG. 1.

similarly diluted with concentrated picric acid solution. In our experiments 2 cc. of each of the three dextrose solutions in 0.8 per cent picric acid were mixed with 2 cc. of Na_2CO_3 solution in water, and 2 cc. of sodium picrate added (prepared by neutralizing 0.8 per cent picric acid with NaOH using phenolphthalein as an indicator). In those cases in which 10 per cent Na_2CO_3 concentrations were used, 3 cc. of Na_2CO_3 solution and only 1 cc. of the picrate were added. Since the picrate is present in excess this change had no appreciable effect on the reaction.

The highest concentration of dextrose employed was thus ten times greater and the intermediate concentration two times greater than our lowest concentration. If the amount of color produced was directly proportional to the amount of dextrose present in the three mixtures the same relationship of 10, 2, and 1 would be reproduced in the colors obtained after heating. In that case if after heating we diluted the mixture with the lowest dextrose concentration to 10 cc., the intermediate one to 20 cc., and the one with the highest concentration to 100 cc., we should find in all three mixtures so diluted the same color, and would obtain the same reading on the Duboscq colorimetric scale.

This was the procedure adopted in all the experiments given here. The results were plotted on a scale of which the ordinate gave the amount of color produced and the abscissa the variation of each factor studied. A curve was thus obtained from each dextrose concentration. That obtained from the highest concentration is represented by solid dots, the intermediate by triangles, and the lowest by circles. The degree to which these curves fail to coincide represents the degree to which the production of color fails to be in direct proportion to the concentration of dextrose.

Effect of Temperature.

The effects of temperature variations were followed under a number of different conditions, but it will be sufficient to illustrate the influence of this factor at three different alkali concentrations, time remaining constant. Figs. 2, 3, and 4 show the effect of temperatures from 100–130°C. with 1, 4, and 10 per cent concentrations of sodium carbonate. These figures refer to the concentrations present in the heated mixtures, except for the slight reduction arising from the acidity of the 2 cc. of 0.8 per cent picric acid in which the dextrose was dissolved.

It will be noted that color production is not directly proportional to dextrose concentration under any of these conditions, for in no case do the curves exactly coincide. The general effect of temperature increase is in all cases to increase the color production, but even at the highest temperature and with the greatest alkali concentration the reaction is incomplete.

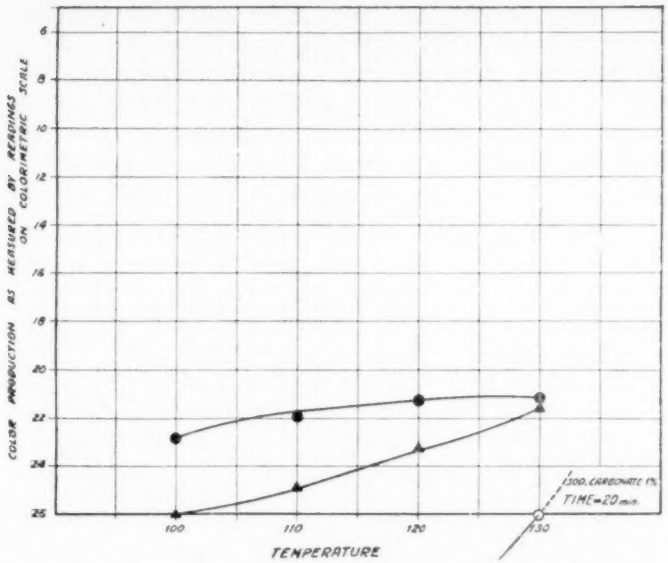

FIG. 2. In this and in all the following charts the highest concentration of dextrose is represented by solid dots, the intermediate by triangles, and the lowest by circles.

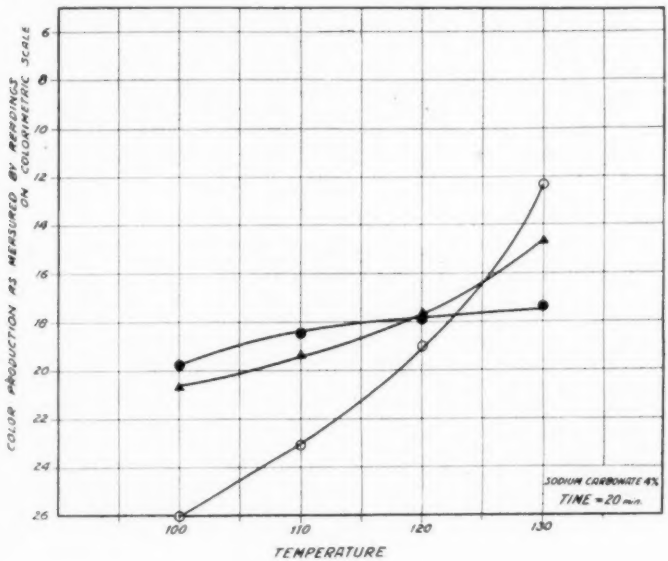

FIG. 3.

The lower the dextrose concentration the greater is the accelerating influence of a rise of temperature, and at alkali concentrations above 1 per cent this leads to a crossing of the curves so that while at 100°C. the amount of color produced is relatively less with low than with high dextrose concentrations, the reverse is true at 130°C.

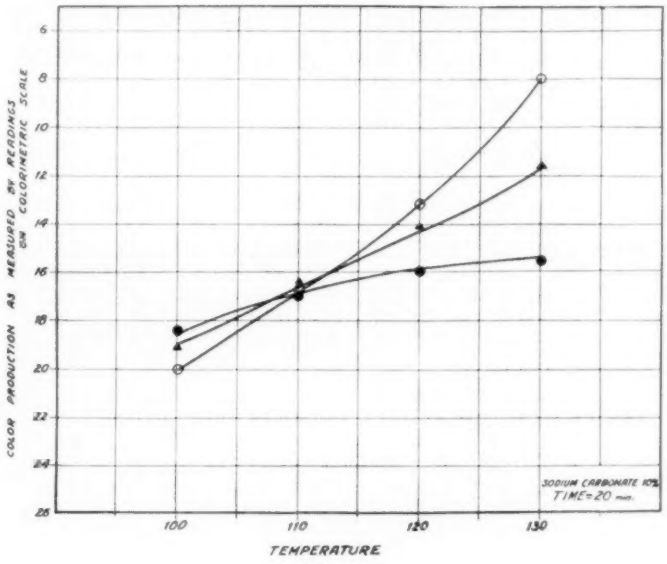

FIG. 4.

Effect of Hydroxyl Ion Concentration.

It is apparent from the temperature curves that the factor of OH ion concentration is of primary importance in the reaction between dextrose and picrate. The influence of this factor was therefore studied in detail at varying temperature levels, time again remaining constant. The effect of variations in Na_2CO_3 concentration from 1 to 10 per cent is given at temperatures of 130, 120, 110, and 100°C. in Figs. 5 to 8.

In none of these graphs is there an instance in which all three curves cross at the same point, and therefore under none of these conditions is color production directly proportional to dextrose

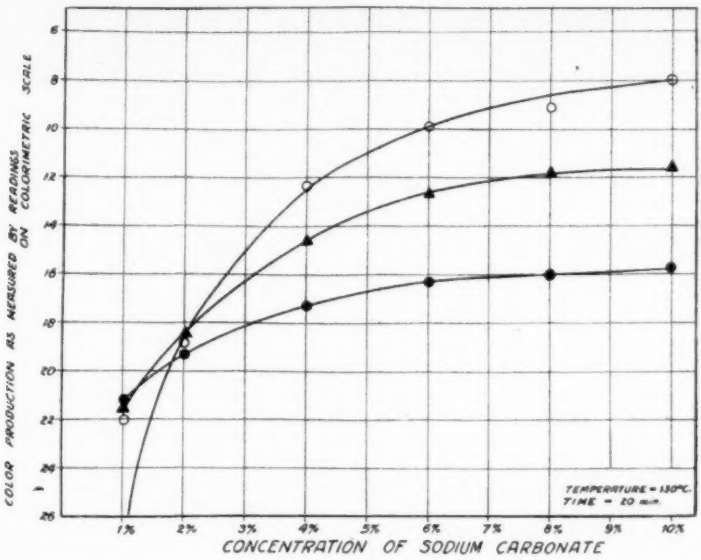
COLOR PRODUCTION AS MEASURED BY READINGS ON COLORIMETRIC SCALE
CONCENTRATION OF SODIUM CARBONATE
TEMPERATURE = 130°C.
TIME = 20 min.

FIG. 5.

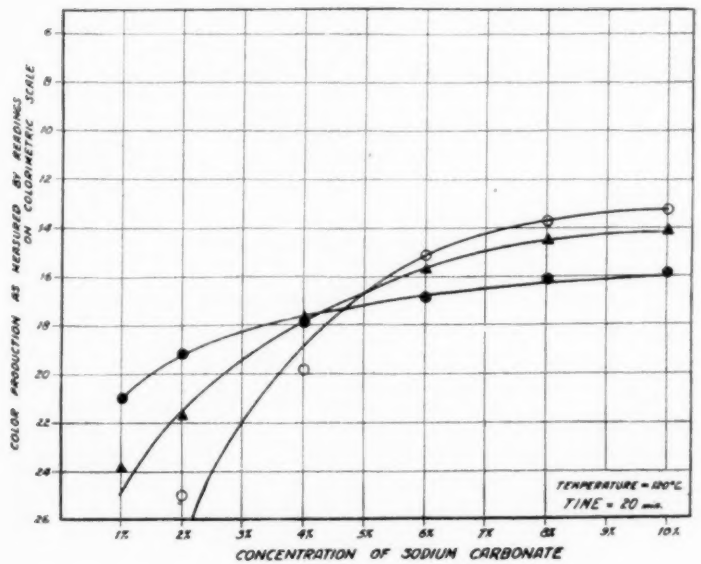
COLOR PRODUCTION AS MEASURED BY READINGS ON COLORIMETRIC SCALE
CONCENTRATION OF SODIUM CARBONATE
TEMPERATURE = 120°C.
TIME = 20 min.

FIG. 6.

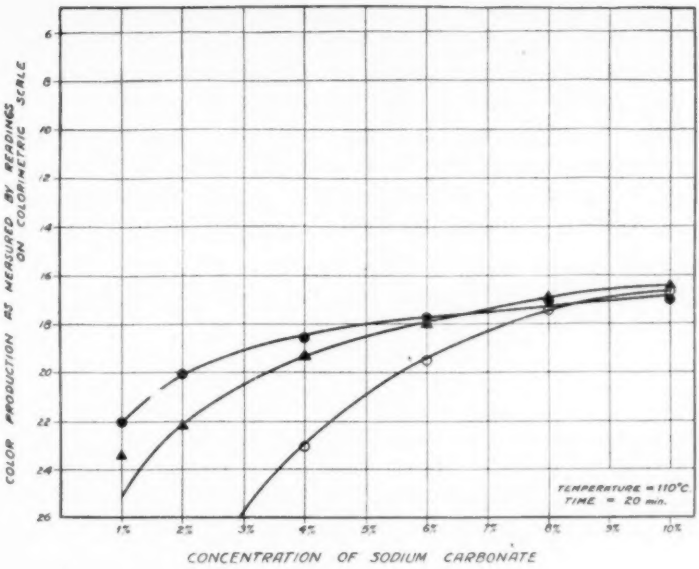
COLOR PRODUCTION AS MEASURED BY READINGS ON COLORIMETRIC SCALE
6
8
10
12
14
16
18
20
22
24
26
1%
2%
3%
4%
5%
6%
7%
8%
9%
10%
TEMPERATURE = 110°C.
TIME = 20 min.
CONCENTRATION OF SODIUM CARBONATE

FIG. 7.

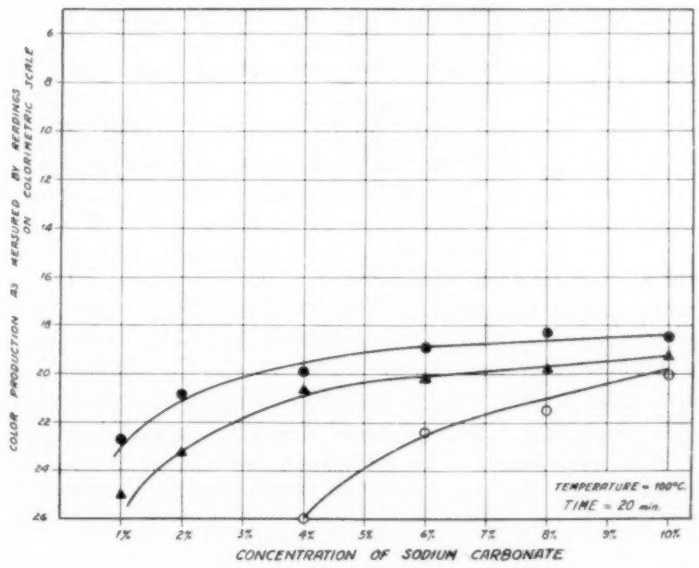
COLOR PRODUCTION AS MEASURED BY READINGS ON COLORIMETRIC SCALE
6
8
10
12
14
16
18
20
22
24
26
1%
2%
3%
4%
5%
6%
7%
8%
9%
10%
TEMPERATURE = 100°C.
TIME = 20 min.
CONCENTRATION OF SODIUM CARBONATE

FIG. 8.

concentration. But in Fig. 8, with a temperature of 100°C. the curves are drawing closer together on more or less parallel planes so that it seemed that if the OH ion concentration could be still further increased they would almost merge with one another. Greater concentrations of Na_2CO_3 were impracticable since sodium picrate crystallizes out at room temperature and makes it impossible to read the color of the mixture with the lowest dextrose concentration which was diluted to only 10 cc.

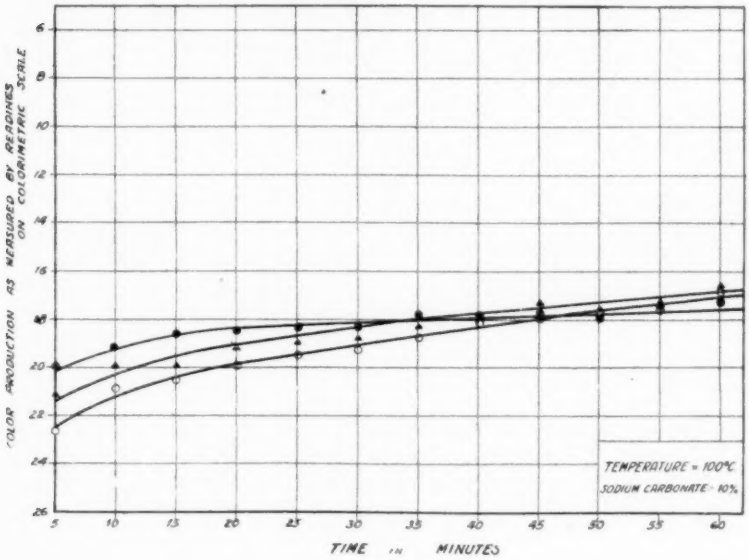

FIG. 9.

Potassium carbonate could not be used because potassium picrate is even less soluble than sodium picrate. Greater OH concentration can, however, be obtained by using NaOH and we plotted curves of the results obtained with concentrations of from 1 to 10 per cent NaOH, but failed to find conditions suitable for the quantitative determination of dextrose concentration.

Effect of Time.

Lengthening the time of heating accentuates the effect of the alkali. When the dextrose solutions were heated at 100°C. with

10 per cent Na_2CO_3 for 45 minutes, the curves almost coalesced. The effect of time under these conditions is shown in Fig. 9. If these curves at 20 minutes are compared with those in Fig. 8 at 10 per cent Na_2CO_3 it will be seen that from that point onwards they represent the continuation of the curves of Fig. 8.

The point of practical significance is that all dextrose concentrations within the range of those derived from blood when heated for 45 minutes at 100°C. in the presence of 10 per cent Na_2CO_3 and 0.8 per cent picric acid will yield an amount of color almost if not quite directly proportional to the concentration of dextrose present.

CONCLUSIONS.

When a solution of dextrose and picrate is heated in the presence of an alkali the rate of production of a color resembling that of sodium picramate increases with increase in the OH concentration and in the degree and duration of heating.

Although increase in dextrose concentration leads to an increase in the rate of color production, yet within a range of dextrose concentrations corresponding to those derived from hypoglycemic, normal, and hyperglycemic bloods the rate of color production is different for each concentration and does not change in direct proportion to the change in dextrose concentration. But when an amount of sodium carbonate sufficient to produce a concentration of 10 per cent is added and the heating is continued for 45 minutes at 100°C. in the presence of 0.8 per cent picric acid, the rate of color production in all dextrose concentrations within the above range becomes almost if not quite directly proportional to the particular dextrose concentrations used.

Addendum.—After this work had been completed we learned that Benedict[1] had arrived at somewhat similar conclusions and at the last meeting of the American Society of Biological Chemists had outlined a method under which the disproportion between color production and dextrose concentration is obviated. But we believe our results are worthy of publication since they are the foundation for a method of blood sugar estimation which we have found satisfactory.

[1] Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiii, p. xviii.

A MODIFICATION OF THE PICRATE METHOD FOR BLOOD SUGAR DETERMINATIONS.

By T. ADDIS and A. E. SHEVKY.

(From the Medical Division of Stanford University Medical School, San Francisco.)

(Received for publication, April 15, 1918.)

We have shown in the preceding paper that there is a certain complex of conditions under which solutions of dextrose in any of the concentrations which may exist in the blood will produce degrees of color from which the actual concentrations may be determined with a considerable degree of accuracy. But this color production depends on a reaction which is always incomplete and which may be markedly affected by slight alterations in the medium. It was, therefore, possible that among the many substances in the blood which are not precipitated by picric acid there might be some which would alter the rate of reaction. Conditions suitable for the determination of dextrose dissolved in water would then not be adapted for blood filtrates.

This question cannot be decided directly. But if the assumption is made that the true dextrose concentration of any blood filtrate may be determined by heating with picrate for 45 minutes at 100°C. with 10 per cent Na_2CO_3 and these filtrates are then treated as known concentrations of dextrose and curves of color production are plotted from the results obtained by heating them at higher temperatures and variable alkali concentration, any error in this assumption will be made apparent by a disagreement between the form of the curves obtained from the filtrates, in which the dextrose concentration has been thus estimated, and the curves obtained from dextrose solutions known to contain these concentrations.

Large amounts of blood filtrates were prepared from normal rabbits and from rabbits rendered hyperglycemic by means of

adrenalin, and curves under varying conditions plotted on the assumption that their dextrose concentrations had been correctly estimated under the conditions suitable for dextrose in water. Within the limits of the technical error of the method these curves were found to agree with those obtained from dextrose solutions of the same concentration.

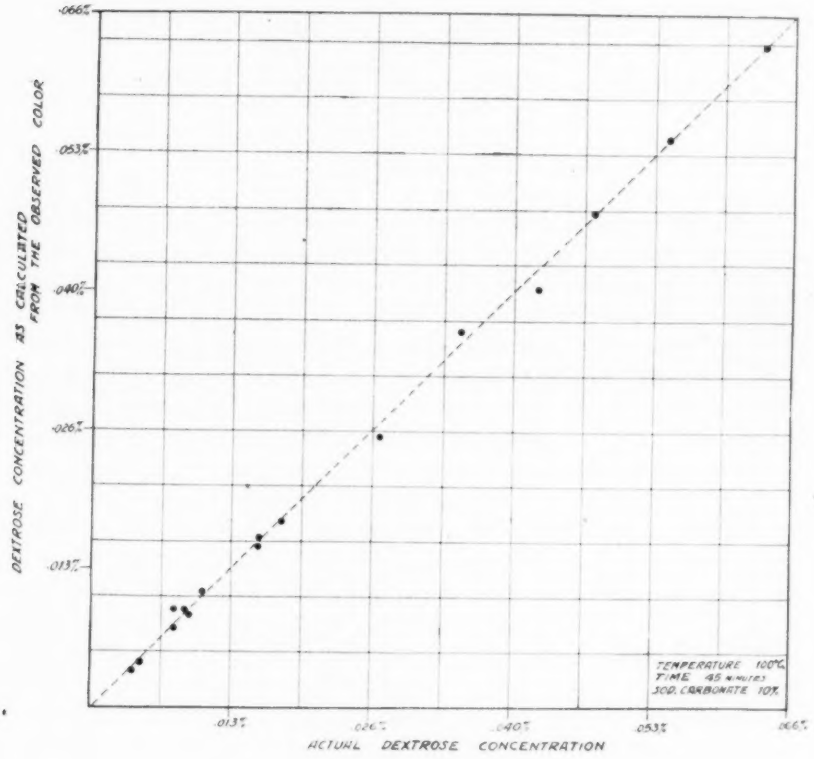

FIG. 1.

If there is any substance in blood filtrates which alters the rate of the reaction one would expect it to have less and less effect with progressive dilution of the filtrate. In that case the colors produced by dilutions of the filtrate would not have the direct linear relationship to one another in accordance with the degree of dilution which is yielded by dilutions of dextrose solutions in

water when they are heated at 100°C. for 45 minutes with 10 per cent Na_2CO_3 and 0.8 per cent picric acid. In Fig. 1 the results of determinations of various dilutions of filtrates from the specimens of hyperglycemic blood are given. The broken line gives the theoretical dextrose concentrations arising from dilution. The actual determinations approximate fairly closely to the theoretical.

There is thus no evidence that picric acid filtrates from rabbit blood contain any substance which alters the rate of color production, and therefore, the conditions found best for the determination of dextrose in water apply also to the dextrose in blood filtrates.

The accurately regulated autoclave we have described is not necessary since the optimum temperature is 100°C. But the conditions existing in an open water bath are not sufficiently uniform. Air currents passing over the tubes while they are being heated induce slight differences in the amount of water evaporating from them, and consequent inequalities in the concentrations of alkali and dextrose which, in a reaction so delicate as this, may be sufficient to lead to appreciable error. These are prevented by covering each tube with a rubber cap through which a small puncture has been made. The caps stretch when the tubes are placed in boiling water and the puncture opens but closes again when the temperature has become uniform.

Variation in the condensation of water on the sides of the tubes is prevented by heating them in a closed vessel. We have found the usual household electrical coffee percolator convenient. The central tube is sealed at the top and holes are drilled along the sides so that the heated water passes out radially in all directions (Fig. 2).

The supply of heat is relatively large so that the water boils vigorously. There is a slight degree of steam pressure which may tend to minimize the effect of changes in barometric pressure. We have not noted any appreciable change in temperature under varying weather conditions. A thermometer whose bulb is in contact with the heated plate at the bottom of the percolator gives a reading of 101°C.

The details of the technique we have used are as follows: The picric acid used was an 0.8 per cent solution in water. The

sodium picrate was prepared by neutralizing this picric acid solution with NaOH in substance, the amount required being determined by titration using phenolphthalein as an indicator. These solutions were kept in dark bottles and frequently renewed. The sodium carbonate was made in 20 per cent concentration

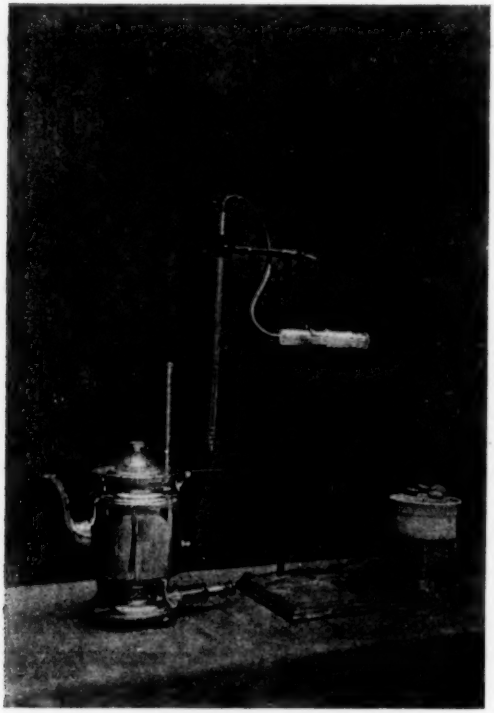

Fig. 2.

from Mallinckrodt's monohydrated sodium carbonate. This solution was standardized by titration to contain 20 per cent of the carbonate without water of crystallization. The picrate was measured from a small micro-burette, and the sodium carbonate from a Thompson pipette shown in Fig. 2. A 2 cc. Ostwald pipette was used for the blood filtrate. It is necessary that all glassware be accurately calibrated.

The blood or plasma was poured into a small weighing glass whose volume capacity had been determined, and which could be completely filled and stoppered without bubbles. It was then emptied into a small test-tube which contained 1.5 times as much picric acid as the volume of the blood or plasma. Picric acid in substance, 0.2 gm. for each 10 cc. of blood or plasma, was thoroughly stirred in with a glass rod. Parts of this mixture were repeatedly poured into the weighing glass and stirred in order to include that part of the blood or plasma which had remained adhering to the sides of the glass. The whole was then centrifuged and the supernatant fluid filtered through cotton wool.

The filtrate was pipetted into test-tubes in 2 cc. volumes, and 1 cc. of sodium picrate and 3 cc. of Na_2CO_3 were added. These quantities were delivered to the bottom of the tubes and not allowed to drain down the sides. The tubes were gently shaken to insure mixing, covered with rubber caps, and kept in the percolator for 45 minutes. After heating they were placed at once in ice water for 10 minutes, then transferred to water at room temperature, diluted, and read in a Duboscq colorimeter.

As a direct proportion between color production and dextrose concentration is not exactly attained, even under these conditions it is necessary that the relation between them be accurately determined. For this purpose various concentrations of dextrose were prepared of such strength that they varied from 0.066 to 0.0066 per cent when mixed with picrate and alkali in the tubes.

The solution produced after heating by the 0.066 per cent concentration was diluted to 100 cc. and the others in direct proportion, 0.033 per cent to 50 cc., 0.0066 per cent to 10 cc., etc. The colors so diluted were read against a sodium picramate standard[1] and the results plotted on a graph whose abscissa gave both the concentration of dextrose and the degree to which it had been diluted.

This standard curve shown in Fig. 3 gives the relation between concentration of dextrose and color production under the conditions here described and is adapted for use in calculating dextrose

[1] The color of this standard was equal to the color produced by heating 28.7 mg. of dextrose dissolved in 2 cc. of picric acid with 1 cc. of sodium picrate and 3 cc. of 20 per cent Na_2CO_3 and afterwards diluting to 50 cc.

concentrations in blood from 0.5 to 0.05 per cent. At least three tubes are prepared from each filtrate.* After heating, one of them is diluted to such a degree as to give a reading between 15 and 20 against the picramate solution mentioned above. From this result a preliminary estimation of the dextrose concentration is made assuming as standard the dextrose concentration on

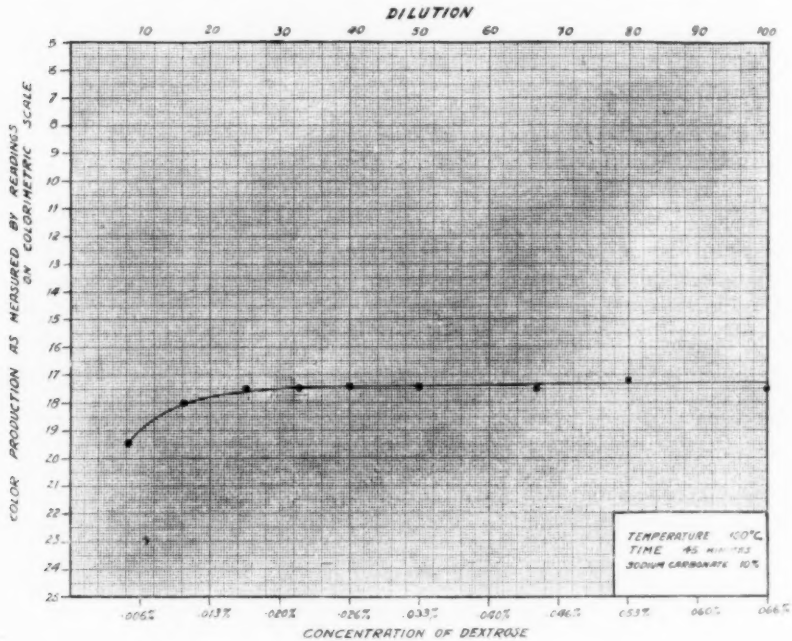

FIG. 3.

the curve which coincides with the dilution used and employing the formula:

$$\frac{\text{Reading of the assumed standard}}{\text{Reading of the unknown}} \times \frac{\text{concentration of dextrose in assumed standard}}{\text{dilution of the assumed standard}} \times \text{dilution of the unknown} = \text{concentration of dextrose in unknown.}$$

This done, it is only necessary to dilute the remaining tubes to the required degree found from the preliminary estimation, and

to recalculate the results after replacing the figures for the assumed dilution by those of a concentration of dextrose nearest to that of the unknown. The results so obtained give the concentration of dextrose in the tubes and since the blood or plasma was diluted to 2.5 times its volume with picric acid in order to remove the protein, and since the 2 cc. of filtrate was diluted to 3 times its volume by picrate and alkali, the percentage concentration found in the tube must be multiplied by 7.5 to obtain the concentration of dextrose present in 100 cc. of the blood or plasma.

Work carried out with this method will soon be reported and the probable error of the determinations will then be given.

CONCLUSIONS.

1. Picric acid filtrates from rabbit blood or plasma contain no substance which alters the rate of color production in heated mixtures of dextrose, picrate, and alkali.

2. A method whereby determination of blood or plasma dextrose concentration may be carried out with simple apparatus is described.

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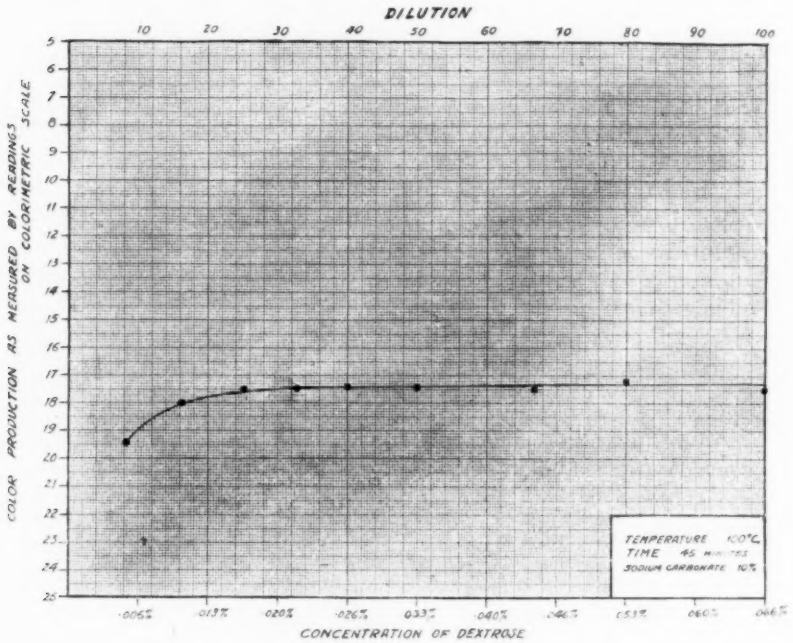

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THE DIETARY QUALITIES OF BARLEY.*

By H. STEENBOCK, HAZEL E. KENT, and E. G. GROSS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, May 23, 1918.)

Due to the pioneer work of Hopkins, Osborne and Mendel, Funk, and McCollum it is now possible, using the rat as the experimental animal, to obtain quantitative data on the specific dietary qualities of many materials used as nutrients. The importance of the standardization of the method of experimentation in work of this nature has been emphasized by the eagerness with which investigators other than those already mentioned have seized upon the opportunity to obtain results applicable not only in the nutrition of animals but of man as well. Already data, though more or less fragmentary in character, have been accumulated in relation to the following materials: maize,[1] wheat,[2] oats,[3] rice,[4] peas,[5] cottonseed meal,[6] navy beans,[7] soy beans,[8] peanuts,[9] wheat embryo,[10] and some animal tissues.[11]

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* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 153; 1918, xxxiii, 303, 411.

[2] McCollum, E. V., Harvey Lecture, *J. Am. Med. Assn.*, 1917, lxviii, 1379.

[3] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 341.

[4] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

[5] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxiii, 411.

[6] Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307; 1917, xxx, 243; 1917, xxxi, 379. Osborne, T. B., and Mendel, L. B., *ibid.*, 1917, xxix, 289.

[7] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.

[8] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91. Osborne and Mendel, *ibid.*, 369.

[9] Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1918, xxxiii, 295.

[10] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.

[11] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxii, 309.

Though the data cover a considerable range of nutrients from diverse sources much remains to be accumulated before the human dietitian or the animal feeder—unless willingly assuming the rôle of a speculator—can state what limiting factors are imposed on an animal by subsistence on a ration compounded from various sources.

In this biological method of analysis results are obtained which are not always directly applicable to animals of different species. For instance, it has lately been found in this laboratory that the requirements of birds for the water-soluble vitamine are far greater than those of the rat.[12] Specific unequivocal data can only be obtained by direct experimentation on the animals in question. In this connection it must not be forgotten that the difference in the anatomical features of the digestive tracts of different animals entirely precludes the possibility of obtaining comparable data of the nutritive value of rations for the ruminant, for example, as compared with the rat. Data on the supplementary value of different concentrates and roughages for milk production—a problem of tremendous importance under present economic conditions—must be obtained by direct experimentation on the dairy cow as carried out by Hart and Humphrey[13] in the course of the last 4 years. As "guiding information" for the outlining of future work much of value can, however, be obtained by the use of the rat as the experimental animal. When once the dietary properties of various naturally occurring foods have been ascertained individually, it will in many cases be possible to forecast with a considerable degree of certainty the nutritive values of combinations of these. In any case the situation is far from being simple as is especially evident in the case of protein supplementation where the eighteen amino-acids must be considered as the physiological units, many of which, by the inability of the body to synthesize them, can act as growth determinants.

In this paper are presented the results of experiments with barley which in themselves may not offer any striking peculiarities, yet they may serve to allay the fears of those dietitians concerned over the use of barley as a wheat substitute. By comparison of these data with such data as are available for maize, oats, and wheat it is indicated that the barley kernel does not differ essentially from these grains in its nutritive properties.

The method of experimentation was essentially that previously used with other materials in this laboratory.[3-6, 9, 12] The whole barley grain was ground to a coarse meal, then dried at 70–80° for 2 hours, and ground up very fine. To this meal were added the various supplements; protein by the addition of casein, fat-

[12] Unpublished data.

[13] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239; 1916, xxvi, 457; 1917, xxxi, 445.

soluble vitamine by the addition of butter fat, and salts in the form of a complete salt mixture or its components. These additions were made singly and in the various possible combinations. In a few instances where it was advisable to add the water-soluble vitamine this was added in the form of a 95 per cent alcohol extract of ether-extracted wheat embryo.

DISCUSSION.

Barley alone is unable to meet the demands of the growing animal or even to allow noteworthy amount of growth to take place. The addition of the fat-soluble vitamine in the form of butter fat improves its growth-promoting quality to some extent, but the addition of casein is of little if any benefit. In fact the animals on this latter ration seemed to be more susceptible to cutaneous infection. Of the single additions, the salt mixture by satisfying the demands of the animal for the elements in the group calcium, chlorine, and sodium exerted the most beneficial influence. When only these were supplied, however, the animals were not able to maintain themselves as long as when the complete salt mixture was added. Gaseous intestinal fermentation with abnormal distention of the tract seemed to be the immediate cause of death. The important rôle of the mineral elements is again brought out when multiple additions were made; only where salts were one of the additions was substantial growth noted. Where all these additions, *i.e.* salts, protein, and fat-soluble vitamine, were made, normal growth, reproduction, and rearing of the young became possible.

Barley contains an abundance of the water-soluble vitamine. Even when the amount of barley was reduced to 60 per cent of the ration it was found possible for a female to rear a litter of young. On 40 per cent of barley growth was entirely normal, but no young were reared. On 20 per cent growth was not quite normal. In an attempt to have the females on this ration raise their young, three litters consisting of a total of fourteen young averaging 5 gm. in weight were reduced to three and left with the nursing mothers. They gained rapidly in weight up to an average of 26 gm., but then started to lose until they averaged but 19 gm. At this time they gave definite evidence in their behavior that they

suffered from a lack of the water-soluble vitamine. They appeared to be paralyzed posteriorly, yet spasmodically without any apparent cause they would dart up the sides of the cage in the most excited manner. For 2 days they were given cow's milk with a medicine dropper in addition to what nourishment they got from their mothers, but with no resultant improvement. They were then put with normal nursing mothers. Improvement was evident by the next day and growth thereafter was rapid. They were kept until they averaged 118 gm. in weight. When the amount of water-soluble vitamine was reduced in amount to that contained in 10 per cent of barley, growth was markedly interfered with. The persistence of the function of reproduction even under these conditions was indicated in the case of Rat 48 which was started on the ration when almost one-third grown. She gave birth to three litters of young. The first litter of four grew rapidly, attaining the combined weight of 62 gm. by the 13th day, but 6 days later they had gained only 4 gm. more. Thereafter they lost weight rapidly, two dying on the 22nd day after showing symptoms indicative of a lack of water-soluble vitamine. The other two were killed by a foster mother who refused to adopt them. The second and third litters were kept only a few days. It is suggestive that while the young of the first litter averaged 5 gm. in weight those of the second averaged 4 gm. and those of the third still less; *viz.*, 3.3 gm. It is, of course, possible that the young of the second and third litters were born prematurely, but as indicated by the curve of weight increase of the mother (Chart 15) this is hardly probable. It is also possible that the size of the litters may have had some influence on the average weight of the young, yet in our colony we have never noted such great differences in weight with successive litters of average size. May it not be more probable that with a deficiency in the water-soluble vitamine so as to interfere with extrauterine growth we may have with a repeated strain of reproduction interference with intrauterine growth as well. When the young of the different litters were born and subsequently, the mother was in excellent nutritive condition. When later transferred to a ration such as is used for our stock colony, this same rat gave birth to a litter of four which averaged over 6 gm. in weight and grew to be normal rats.

While barley contains an abundance of the water-soluble vitamine, it is deficient in the fat-soluble vitamine. This was indicated by the improvement in growth when butter fat was added and by the fact that some of the rats on rations without fat-soluble vitamine additions developed a purulent conjunctivitis. When all requirements other than that for fat-soluble vitamine have been provided for, the amount of fat-soluble vitamine introduced with 15 per cent of barley, so far as improvement in growth is concerned, is negligible. 60 per cent, however, improves the ration markedly. The deficiency in this factor cannot be very great, yet it is sufficient to be a factor of importance, especially when intensified in action by other deficiencies.

The protein content of barley (13.6 per cent) is too low for continued growth at the normal rate. When reduced in amount to 8.1 per cent, the small amount of growth that results is soon followed by a decline. 5.4 per cent just about suffices for maintenance.

The primary growth determinant in barley is inorganic salts. Of secondary importance, but no less urgent, are protein and fat-soluble vitamine.

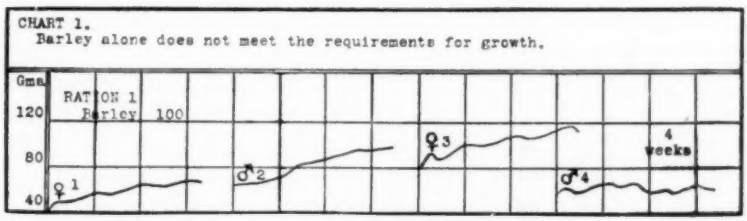

CHART 1. Ration 1. Young rats are unable to grow on barley as the sole source of nutrients. All of these rats ultimately became afflicted with multiple infections of the ears and tails which in some cases gave indications of a tendency to spread to the legs and other parts of the body. Such infections, as we are inclined to consider them, can be cured by treatment with tincture of iodine. The deficiencies in the ration are the predisposing factors as rats on good rations are with few exceptions not subject to them and those on poor rations show gradual improvement upon making suitable dietary modifications.

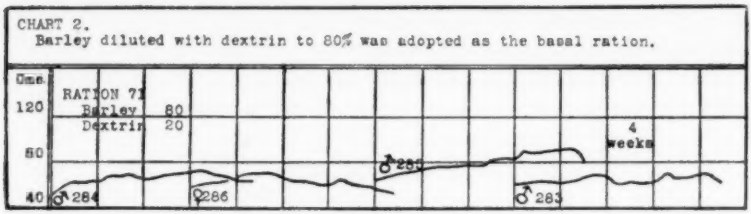

CHART 2. Ration 71. Growth on 80 per cent of barley as on barley alone (Chart 1) is impossible. The 20 per cent of dextrin in the ration has no special physiological properties except to furnish energy. By substituting various constituents for part or all of the dextrin, the modifications necessary to make barley a complete food are later brought out.

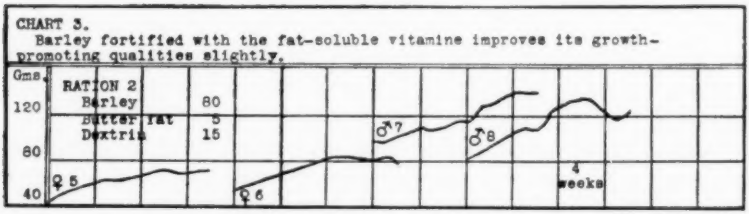

CHART 3. Ration 2. Butter fat, rich in the fat-soluble vitamine, is not able to cover the deficiencies in the nutrients obtaining in barley. The growth performance of these animals is a little better than on 80 per cent of barley without supplements. These animals were just as subject to infections as those not receiving additional amounts of the fat-soluble vitamine.

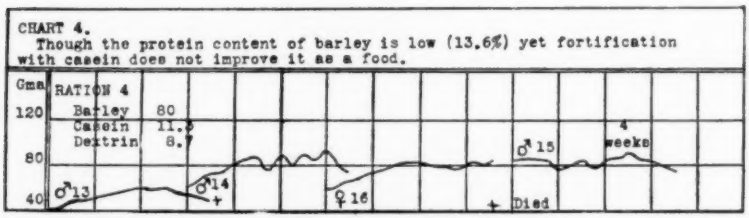

CHART 4. Ration 4. When barley is supplemented with casein no improved condition, as indicated by the failure of the experimental animals to grow, is apparent. In fact, none of the rats on any of the other rations of this series went into such a miserable condition as these; a high protein ration in the absence of other essential factors of proper alimentation aggravates the condition of heightened susceptibility to multiple infections.

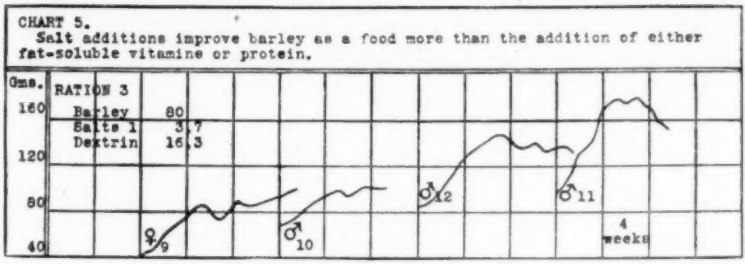

CHART 5. Ration 3. Salts alone of the single additions of the important dietary factors to barley are able to bring about the utilization of nutrients otherwise latent in their relation to satisfying the growth impulse. Mineral elements are the primary growth determinants.

The salt mixture used, Salt Mixture 1, was a complete salt mixture which has been used in this laboratory by McCollum. It is far more complex than necessary but the possibility of direct comparison of these experiments with previous results was thought to make its use advisable. It had the following composition:

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4.H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Iron citrate	0.118
Calcium lactate	1.300

This mixture was dried at 95° and then ground. Due to loss of water in drying, the amount of the above mixture actually incorporated with 3.7 parts in 100 gm. of the ration was 4.3 parts.

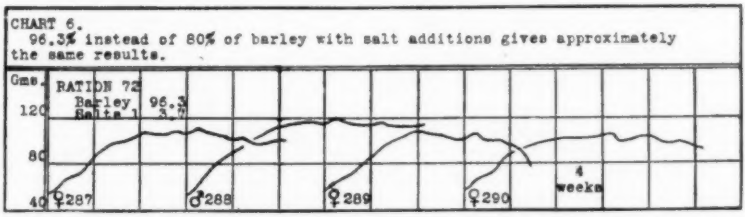

CHART 6. Ration 72. When barley alone is supplemented with salts, the additional amounts of the essential dietary factors, furnished by the 16.3 per cent of barley, do not alter the situation indicated in Chart 5, Ration 3. Growth for any long period of time even with this addition is not possible and even maintenance ultimately fails. Rat 288 after 9 weeks on this ration became afflicted with edematous eyes indicative of a probable deficiency of the fat-soluble vitamine.

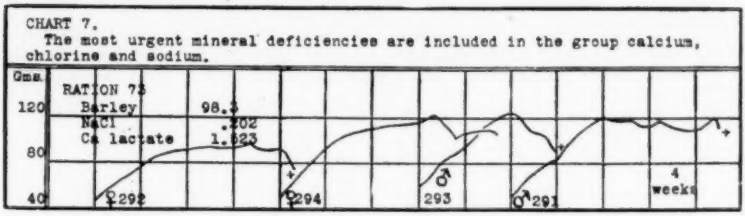

CHART 7. Ration 73. Barley supplemented with sodium chloride and calcium lactate was not as good a ration as when supplemented with a complete salt mixture. Three of the rats died within 4½ months after being started on this ration. Postmortem examination revealed constipation with gaseous fermentation.

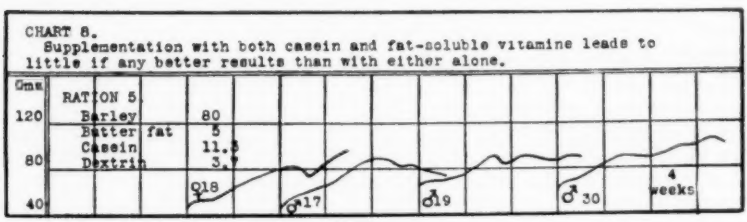

CHART 8.

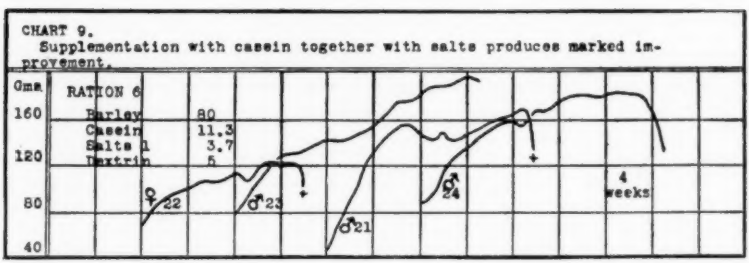

CHART 9. Ration 6. When barley is supplemented with a complete salt mixture in addition to casein, the accentuated untoward effects of casein supplementation alone are mitigated. Growth, though irregular, occurs. Supplementation is far from complete, and death ultimately supervenes.

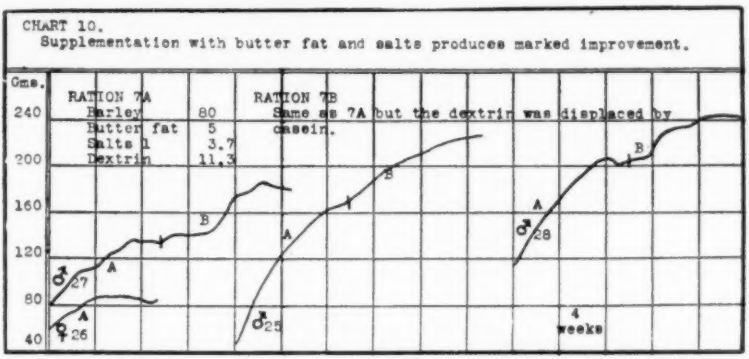

Chart 10. Rations 7A and 7B. Supplementation with butter fat and salts leads to little better response in growth than supplementation with casein and salts. That the low protein content (10.9 per cent) is now the growth determinant is seen when Ration 7B was fed. With the addition of 11.3 per cent casein, as well as salts and butter fat, none of the harmful effects of a high protein diet so evident in Ration 4 were observed. With complete supplementation the protein is metabolized normally and utilized in the building up of new structures.

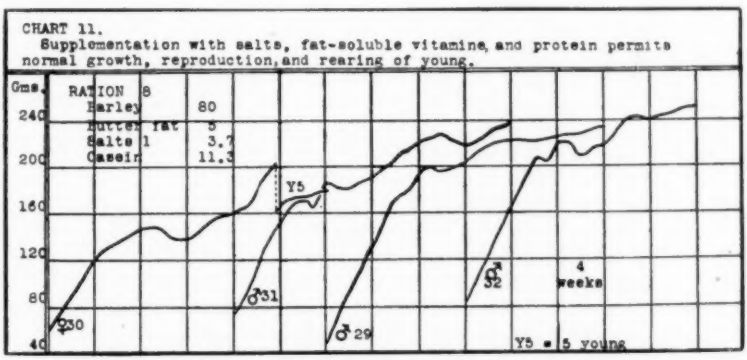

Chart 11. Ration 8. While no additions of any two dietary factors were able to make barley a complete food (Charts 8, 9, 10) additions of three, *viz.* protein, salts, and fat-soluble vitamine, made normal growth, reproduction, and even rearing of the young possible. Rat 30, while its growth curve was irregular, gave birth to five young of which three were reared in 29 days to an average weight of 42 gm.

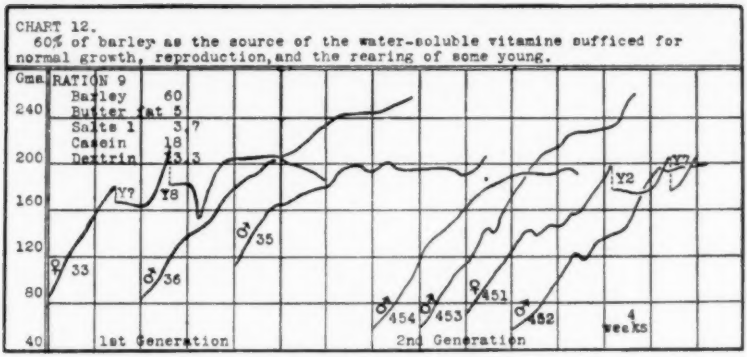

CHART 12. Ration 9. In all the rations so far presented no less than 80 per cent of barley furnished the water-soluble vitamine. On this ration containing 60 per cent of barley, Rat 33 gave birth to two litters of young. The first was disposed of before it could be weighed, but the second consisting of eight young after being reduced to four was raised in 36 days to a total weight of 210 gm. Continued on the mother's ration these also grew normally, but they failed to raise their young.

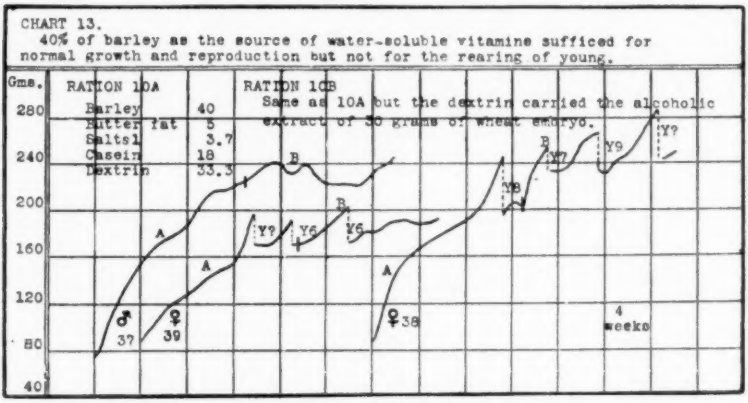

CHART 13. Rations 10A and 10B. Growth and reproduction were entirely normal on this low level of water-soluble vitamine, but no young were reared. Two young from the first litter of Rat 38 were raised to a combined weight of 47 gm. in 18 days, but 6 days later they had been disposed of by the mother. Cannibalism in a non-vicious normal nursing rat is never indicated unless the young are dead or enfeebled. The liberal addition of water-soluble vitamine in the form of an alcoholic extract of ether-extracted wheat embryo did not turn failure into success. A number of factors may have been responsible for this, but we are not certain which it is.

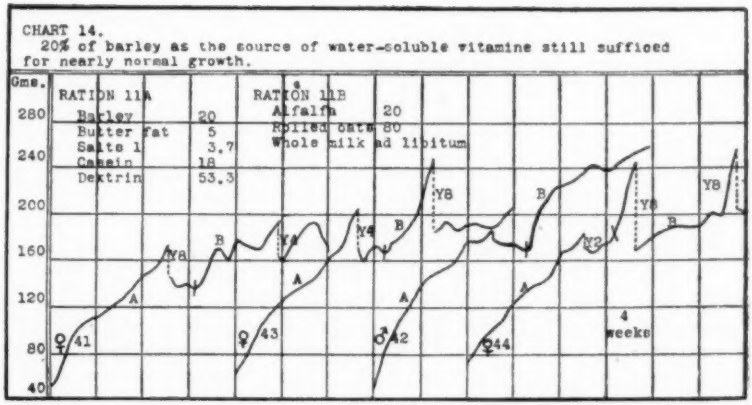

CHART 14. Rations 11A and 11B. While growth was practically normal and reproduction was entirely normal, rearing of the young as on Ration 10A (Chart 13) was impossible on account of a too low water-soluble vitamine content. This was definitely indicated by symptoms observed in the young. Rats 41, 43, and 44 had in their first litter a total of fourteen young weighing 71 gm. These were entirely normal. They were reduced in number to three which the three mothers in 16 days raised to a total weight of 72 gm. 5 days later they had dropped in weight to 58 gm. At this time they appeared as though partially paralyzed, especially posteriorly, yet spasmodically they would suddenly rush around and dart up the sides of the cage. Artificial feeding with fresh cow's milk for 2 days, in addition to what nourishment was provided by the mothers, did not induce any improvement; they still showed intermittent periods of hypersensitivity. After being with foster mothers for 4 weeks, they collectively weighed 298 gm. and appeared to be entirely normal rats. Female rats on a water-soluble vitamine-poor ration are able to produce viable young but are unable to nourish them properly. When changed to Ration 11B in which this dietary deficiency did not exist, each of the three females produced a litter of young which was reared successfully.

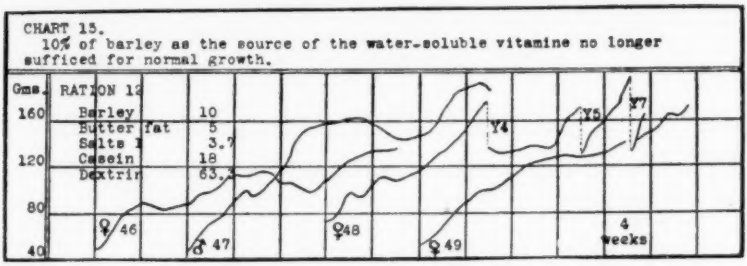

CHART 15. Ration 12. Though normal growth was no longer possible, one female, Rat 48, started on this ration when almost one-third grown, was able to produce consecutive litters of young and showed a remarkable ability to nourish the first litter. It raised a litter of four weighing 20 gm. to a total weight of 62 gm. in 13 days. 6 days later they weighed only 4 gm. more. Thereafter they lost weight rapidly, two dying on the 22nd day when they showed symptoms identical in character with the young of the females on Ration 11. The other two were killed by a foster mother who refused to adopt them. Later, Rat 48 had two more litters, neither of which it kept longer than a few days. When changed over to a mixed ration such as is used for the laboratory stock, it had a fourth litter which was raised in normal manner. The previous severe regimen had not interfered permanently with the function of normal milk production.

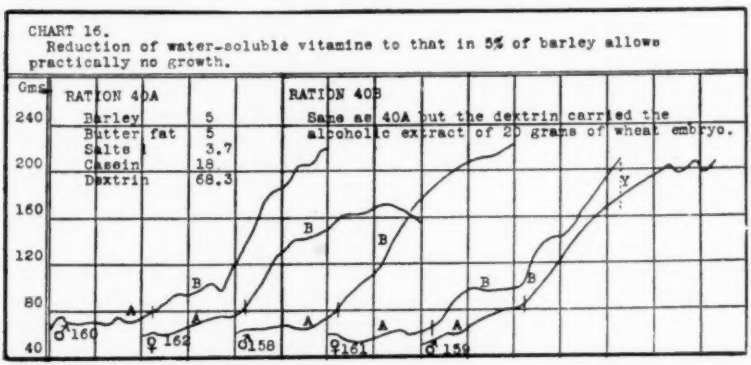

CHART 16. Rations 40A and 40B. Correction of the dietary deficiency of water-soluble vitamine in Ration 40A by the addition of the alcoholic extract of ether-extracted wheat embryo caused prompt resumption of growth. This is not as sharply indicated as is usually the case because the rats were on a slight uphill grade in one of the waves of weight fluctuations before the change of rations was made. The young of one of the females were disposed of by the mother before a record of them could be obtained.

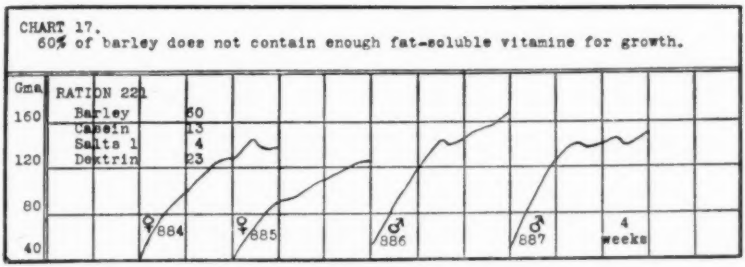

CHART 17. Ration 221. While the fat-soluble vitamine is not the primary growth determinant in barley, yet a deficiency of it was indicated in Rat 288 (Chart 6, Ration 72) which suffered from a conjunctivitis. In this ration we have an interference with normal growth which dietetically can have been caused only by a deficiency of the fat-soluble vitamine. All the other requirements as demonstrated in the previous charts had been met.

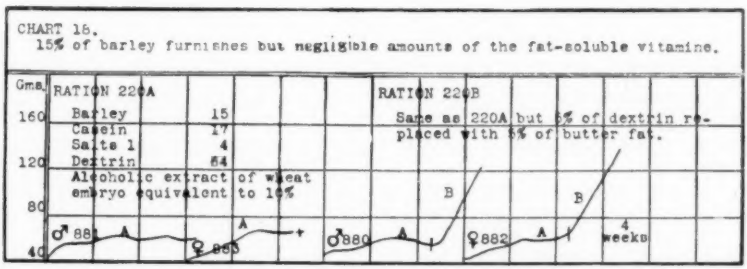

CHART 18. Rations 220A and 220B. On the addition of fat-soluble vitamine in butter fat prompt resumption of growth occurred; all other dietary requirements had previously been met.

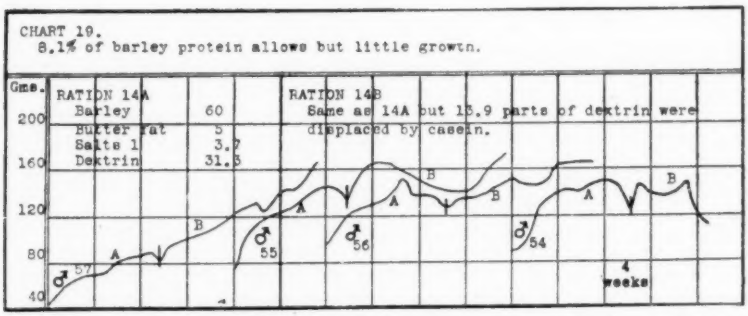

CHART 19. Rations 14A and 14B. On 8.1 per cent of barley protein as compared with 10.9 per cent in Ration 7A (Chart 10) the amount of growth is far less. In this ration the growth impulse inhibited by the low protein diet did not readily manifest itself when the protein content was increased by the addition of casein.

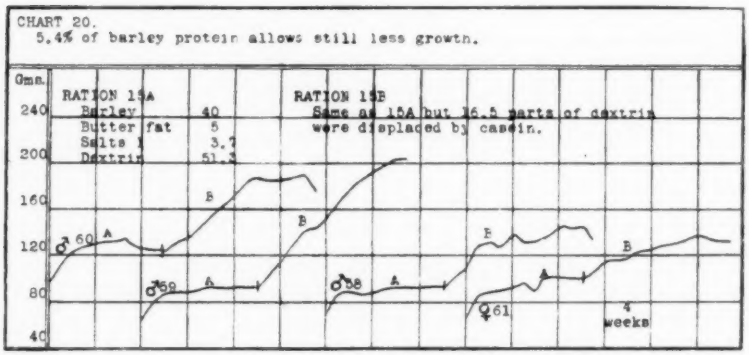

CHART 20.

THE ORIGIN OF CREATINE. III.

By L. BAUMANN and H. M. HINES.

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(Received for publication, May 13, 1918.)

During the past few years Thompson[1, 2, 3] reported numerous injection experiments with arginine on rabbits, ducks, and dogs. In most instances he was able to observe an increase in the elimination of creatine in the urine and in some an increase in muscle creatine as well. Myers and Fine[4] found that the creatine content of the muscle of rats was slightly increased as a result of feeding with edestin, a protein relatively rich in arginine. In our experiments we were unable to obtain decisive results with arginine. To be sure, an increase of creatine in the urine followed one injection, but a similar result was obtained in the control histidine experiment. Riesser[5, 6] studied the effect of injections of choline, betaine, and sarcosine on the creatine content of rabbit muscle and urine. In each case the substance was administered with urea. This investigator believes that all three substances may produce an increase in the creatine content of rabbit muscle. Thompson[3] however, did not observe an additional increase in the creatine elimination when betaine or choline was administered with arginine, nor did the feeding of sarcosine with guanidine increase the creatine output. The theoretical considerations which led to the above experiments are discussed by Riesser.[5]

We have had occasion to perfuse several of these substances through dog muscle. While the results varied somewhat, positive indications were obtained with choline and sarcosine in several experiments. However the magnitude of the increments and the possibility of experimental errors are such that we deem it unwise to assign the rôle of creatine precursors to these substances. It appears to us that caution must be observed in in-

[1] Thompson, W. H., *J. Physiol.*, 1917, li, ii.
[2] Thompson, *J. Physiol.*, 1917, li, 111.
[3] Thompson, *J. Physiol.*, 1917, li, 347.
[4] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 389.
[5] Riesser, O., *Z. physiol. Chem.*, 1913, lxxxvi, 415.
[6] Riesser, *Z. physiol. Chem.*, 1914, xc, 221.

terpreting these and similar experiments until considerable more evidence has accumulated. At present, we wish to record the results without committing ourselves to a definite conclusion.

Methylguanidine has been tested a number of times by former investigators.[7] In all instances the results have been negative. In our experiment the muscle of a dog that had been placed on a creatine-free diet for several months (preceding the experiment) was analyzed before and after the injection of methylguanidine. No increase in muscle creatine was found. Perfusion experiments with this substance were unsuccessful owing to its powerful constricting effect upon the blood vessels.

Volhard[8] synthesized creatine from cyanamide and sarcosine. Jaffé[9] found that glycocoll administered with cyanamide did not neutralize its toxic action. Owing to its poisonous effect our injection experiments with cyanamide were inconclusive.

EXPERIMENTAL.

Arginine.

Experiment 1.—A dog weighing 7 kg. was fed each day with 220 cc. of fresh milk and 160 gm. of dried bread. The urine was obtained by means of a catheter. The bladder was washed with boric acid solution. The arginine and histidine were dissolved in 40 cc. of water and neutralized with acetic acid.

[7] Burns, D., *Biochem. J.*, 1916, x, 263.
[8] Volhard, J., *Sitzungsb. koenigl. bayer. Akad. Wissensch.*, 1868, ii, 472.
[9] Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.

TABLE I.

Effect of Arginine and Histidine Injections on the Composition of the Urine.

Day.	Volume.	Creatinine.	Creatine.*	Total N.	Urea N.	NH_2N
	cc.	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*
1	525	167	9	3.801	3.075	0.221
2	500	160	6	3.925	3.217	0.206
3	400	159	2	3.320	2.633	0.149
4	400	160	8	3.708	3.062	0.201
5	400	165	13	3.231	2.655	0.145
6	400	150	6	3.030	2.469	0.104
7	410	165	2	3.542	2.811	0.208
8	400	150	2	3.120	2.562	0.151
9†	400	167	1	2.820	2.278	0.161
10	470	159	15	3.412	2.715	0.222
Average		160.2	6.4	3.391	2.748	0.177
11‡	545	161	9	3.643	2.924	0.181
12‡	400	160	29	3.788	3.157	0.144
13	410	172	11	3.335	2.773	0.146
Average		164	16	3.588	2.951	0.157
14	415	167	5	3.181	2.539	0.148
15	410	175	3	3.375	2.799	0.163
16	410	165	15	3.283	2.802	0.140
17	450	170	13	3.525	2.947	0.210
18	400	169	8	3.153	2.560	0.156
Average		169	9	3.303	2.729	0.163
19§	400	162	13	3.206	2.664	0.144
20§	400	172	15	3.744	3.052	0.179
21	400	171	6	2.772	2.269	0.125
Average		168	11	3.240	2.661	0.149

* Creatine expressed as creatinine.

† The food was not entirely consumed.

‡ Injection of 2 gm. of arginine.

§ Injection of 1.6 gm. of histidine.

Experiment 2.—A bitch weighing 7 kg. was fed with 220 cc. of fresh milk and 160 gm. of dried bread each day.

TABLE II.

Showing the Effect of Arginine Injections on the Composition of the Urine.

Day.	Volume.	Creatinine.	Creatine.*	Total N.	Urea N.	NH_3N
	cc.	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*
1	400	180	5	3.889	2.974	0.280
2	400	189	20	3.396	2.791	0.183
3	400	162	14	3.301	2.623	0.190
4	400	178	14	3.578	2.886	0.171
5	410	172	9	3.478	2.758	0.155
6	400	164	25	3.573	2.966	0.166
Average		174	14.5	3.536	2.833	0.191
7†	560	178	14	4.233	3.087	0.210
8†	405	178	4	3.780	3.021	0.194
9	405	191	6	3.456	2.833	0.172
10	410	192	3	3.803	3.133	0.185
Average		185	7	3.818	3.018	0.190

* Creatine expressed as creatinine.

† 2 gm. of arginine injected. No toxic symptoms.

Experiment 3.—The liver of a dog weighing 25.4 kg. was ligated with a rubber tourniquet and a portion removed for analysis. The remainder was perfused for 1 hour *in situ* with a solution containing 2 gm. of arginine, 1.56 gm. of KH_2PO_4, 25 cc. of 0.9 per cent NaCl solution, and 255 cc. of defibrinated dog blood. The pressure of the perfusate ranged between 40 and 60 mm. of mercury. The technique of perfusion was described in a previous article.[10] Throughout this paper the creatine content of the liver or muscle is expressed in terms of mg. per gm. of dry tissue.

	Perfusate.		Liver.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	270	14.8	75.02	1.13
After "	280	14.5	74.36	0.95

[10] Baumann, L., and Marker, J. I., *J. Biol. Chem.*, 1915, xxii, 49.

Sarcosine.

Sarcosine with urea was perfused once through the liver and four times through the muscle of dogs.

Experiment 1.—The leg of a dog weighing 14.5 kg. was perfused with defibrinated blood containing 4 gm. of sarcosine and 4 gm. of urea for 1 hour. The entire limb weighed 2,625 gm. while the muscle weighed 1,250 gm.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	225	12.3	73.83	16.01
After "	255	10.6	72.07	15.50

Experiment 2.—The leg of a dog weighing 27 kg. was perfused for 1 hour with a mixture containing 30 cc. of 0.9 per cent sodium chloride solution, 4 gm. of sarcosine, 4 gm. of urea, and 250 cc. of defibrinated dog blood. The entire leg weighed 1,930 gm. and its muscular tissue 1,135 gm.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	280	12.6	67.48	11.75
After "	200	12.3	66.42	11.40

Experiment 3.—The leg of a dog weighing 9.09 kg. was perfused for 1 hour with a solution containing 2 gm. of sarcosine, 2 gm. of urea, 25 cc. of 0.9 per cent NaCl solution, and 255 cc. of defibrinated blood. The leg weighed 682 gm. in its entirety and the muscle weighed 454 gm.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	250	11	71.42	12.61
After "	250	10.6	72.39	12.87

Experiment 4.—The hind limb of a dog weighing 15.9 kg. was perfused with defibrinated blood containing 3 gm. of sarcosine and 3 gm. of urea for 1 hour.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm*
Before perfusion	175	15.1	72.82	13.17
After "	225	12.7	74.46	14.44

Experiment 5.—The left lobe of the liver of a dog was ligated and removed for analysis. The rest of the organ was perfused for 1 hour with a solution containing 150 cc. of defibrinated blood, 25 cc. of 0.9 per cent sodium chloride solution, 2 gm. of sarcosine, and 1.5 gm. of urea.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	200	11.2	67.36	0.705
After "	150	13.2	69.70	0.782

Betaine.

The hind leg of a dog weighing 24.1 kg. was perfused for 1 hour with a mixture of 200 cc. of defibrinated blood, 25 cc. of 0.9 per cent sodium chloride solution, 3 gm. of betaine hydrochloride, and 3 gm. of urea. The betaine and urea were dissolved in the salt solution and neutralized with sodium hydroxide using phenolphthalein as an indicator. The total weight of the limb was 2,273 gm., that of the muscle alone was 1,477 gm.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	225	14.3	73.47	14.02
After "	230	14.1	73.40	14.10

Choline.

Experiment 1.—The hind leg of a dog weighing 19.5 kg. was perfused for 1 hour, with defibrinated blood, to which portions of a neutralized solution of choline chloride and urea (each 3 gm.) were added from time to time. This procedure was adopted to avoid the pronounced constricting effect of choline on the blood vessels.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	275	10.6	72.78	14.84
After "	300	11.5	73.78	14.68

Experiment 2.—The hind leg of a dog weighing 24.5 kg. was perfused for 1 hour with defibrinated blood to which were added 40 cc. of a neutralized watery solution of choline chloride (3 gm.) and urea (4 gm.) as in the previous experiment.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	265	13.2	73.34	14.1
After "	220	11.2	72.98	14.54

Experiment 3.—The hind leg of a dog weighing 29.3 kg. was perfused for 1 hour with defibrinated blood, to which small quantities of a neutralized solution of choline chloride (3 gm.) and urea (3 gm.) dissolved in 25 cc. of water were added from time to time.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion		13.4	71.01	11.93
After "		13.5	71.18	12.52

Experiment 4.—The hind leg of a dog weighing 8.4 kg. was perfused for 1 hour with defibrinated blood to which small quantities of a neutralized solution of choline chloride (3 gm.) and urea (3 gm.) in 25 cc. of water were added from time to time. The weight of the entire limb was 625 gm., that of the muscle alone 400 gm.

	Perfusate.		Muscle.	
	Volume.	Creatine.	Water.	Creatine.
	cc.	*mg. per cent*	*per cent*	*mg. per gm.*
Before perfusion	180	13.4	70.9	13.16
After "	210	11.3	71.98	13.77

Methylguanidine.

A sample was removed from the extensor muscles of the right hind leg of a bitch weighing 14.5 kg. and analyzed for water and creatine. The diet of the animal for months before the experiment had consisted

of lard, bread crumbs, and skimmed milk powder. 2 weeks after the operation small quantities of a solution containing methylguanidine nitrate (9.3 gm.) and glycocoll (5.4 gm.) in water (100 cc.) were injected subcutaneously during a period of 5 days. After each injection the animal became restless, frothed from the mouth, and occasionally had diarrhea. The dog was killed after the last injection and a portion of muscle from the left hind leg, exactly corresponding to that removed for the control, was analyzed for creatine and water. The water content of the muscle before and after the injection was 68.84 and 71.19 per cent, respectively. The creatine content of the dry muscle was 9.8 mg. per gm. both before and after the injection.

Cyanamide.

A bitch weighing 7.3 kg. was placed on a daily diet consisting of 220 cc. of fresh milk and 200 gm. of dried bread. The urine was obtained quantitatively by means of a catheter.

TABLE III.

Effect of the Injection of Cyanamide on the Composition of the Urine.

Day.	Volume.	Creatinine.	Creatine.	Total N.	Urea N.	NH_2N
	cc.	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*
1	400	165	2	3.500		0.189
2	400	164	18	4.088	3.428	0.240
3	415	172	4	3.960	3.324	0.206
4	425	163	25	4.359	3.580	0.198
5	400	167	6	4.438	3.614	0.208
6	400	168	20	4.491	3.740	0.202
7	400	172	8	4.588	3.850	0.182
Average		167	12	4.203	3.589	0.203
8*	550	160	59	4.900	3.950	0.168
9†	400	143	61	4.200	3.384	0.280
10†	470	163	20	4.717	3.860	0.293
11†	400	160	22	4.438	3.388	0.252
12†	400	165	8	4.340		
13†	400	165	21	4.500		
14†	485	173	12	4.331		
Average		161	29	4.489		

* Injected 150 mg. of cyanamide. This was followed by vomiting and other toxic symptoms.

† Injected 50 mg. of cyanamide. Even the smaller doses exerted a toxic effect, as manifested by anorexia and occasional vomiting.

We are indebted to Dr. J. I. Marker for his assistance with the perfusion experiments.

THE EFFECT OF VARIATIONS IN THE AVAILABLE ALKALI ON THE YIELD OF ACETONE IN THE OXIDATION OF BUTYRIC ACID WITH HYDROGEN PEROXIDE.*

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This report concerns oxidations of butyric acid and acetone in the presence of varying quantities of alkali and of glucose, the experiments having been devised with reference to the problem of acidosis of the diabetic or starvation type and of so called antiketogenesis.

Dakin[1] showed that ammonium butyrate may be oxidized by hydrogen peroxide in faintly alkaline solutions to yield, along with products of oxidation at the α-carbon atom, other products: namely, acetone and probably acetoacetic and β-hydroxybutyric acids due to oxidations confined to the β-atom. Neubauer[2] made a similar observation using potassium persulfate as the oxidizing agent, whereas it is commonly held that oxidations of the fatty acids *in vitro* take place as a rule only at the α-atom. Thus under certain conditions butyric acid only gives proof of susceptibility to oxidative attack at the α-atom and under others at both the α- and β-atoms. It might be that the primary oxidative attack on the butyric acid molecule is the same in each case but that the evidences of β-oxidation are lost under the conditions of most laboratory oxidations. Another possibility is that the α- and β-atoms in butyric acid manifest varying degrees of susceptibility to oxidation under varying physical chemical condi-

* The work reported in this article was done in relation to the "Studies on the Theory of Diabetes" being carried out under the direction of R. T. Woodyatt in this laboratory.

[1] Dakin, H. D., *J. Biol. Chem.*, 1908 iv, 77.

[2] Neubauer, O., *Deutsch. Arch. klin. Med.*, 1908–09, xcv, 211.

tions. In the present experiments the attempt was made to ascertain whether the point of oxidation may be shifted by varying the conditions of the experiment. Attention was given chiefly to two variables—alkalinity and quantity of glucose—which were chosen because of the following biological consideration.

1. The so called acetone bodies are commonly assumed to arise in the organism from butyric acid by oxidations at the β-atom. Their occurrence in abnormal quantities in the blood and urine is accompanied by a state of acidosis and the question arises as to the relationships which exist between alkali deficits and abnormal accumulations of β-oxidation products of butyric acid in the body fluids.[3] It is therefore of interest to ascertain whether an alkali deficit is fundamentally favorable to the greater formation of acetone bodies from butyric acid.

2. In connection with the well known power of carbohydrate feedings to lessen or annul the acidosis of fasting, diabetes, etc., the question arises as to how glucose in the course of its metabolism prevents all outward expression at least of β-oxidations. Is the so called antiketogenic effect of carbohydrate the expression of a purely quantitative decrease of the fatty acid combustion, a "sparing" effect of fat by sugar, or is it due to a qualitative alteration of the type of fatty acid oxidation, such, for

[3] It is undeniable that alkali may be administered to individuals with ketosis until the urine is alkaline and the hydrogen ion concentration and the bicarbonate reserve of the blood plasma are normal and that ketosis may still be present without acidosis in the sense that many now use this term. This is true, but it does not follow because a normal reserve of bicarbonate is observed in the blood plasma taken as a whole that there is no deficit of alkali anywhere in the body. There may still be a shortage of effective alkali in the zones of chemical reaction within the cells where oxidations of fatty acids are going on.

In an earlier paper from this laboratory (Woodyatt, R. T., *J. Biol. Chem.*, 1915, xx, 129) emphasis was laid on the parallelism between the behavior of sugars in solutions of higher or lower alkalinity and their behavior in milder or more severe cases of diabetes, respectively. The work of Murlin and Kramer and their associates—showing apparently that alkali feedings may increase the utilization of sugar in diabetes—harmonizes with this view. Nor does the fact that alkali feedings do not increase carbohydrate utilization more than they do, constitute a valid objection to the conception that the normal utilization of glucose depends on alkali or its equivalent.

instance, as a shift in the center of attack away from the β-atom and toward the α-atom? In either case is it, as some have supposed, a direct and purely chemical interaction between *oxidizing* glucose and oxidizing fatty acids, in which case it should be possible to duplicate it by oxidizing butyric acid and glucose simultaneously in the same solution? Or is it an effect which involves and depends upon changes in the cell architecture and one therefore which could not be duplicated *in vitro* in a simple homogeneous system? The experiments to be described were designed to give some light on these questions.

Owing to the great difficulty of analyzing quantitatively a mixture such as is formed in these oxidations, only the acetone was determined quantitatively. This was done on the basis of the assumption that the amount of acetone present is a relative index of the amount of other acetone bodies also present; *i.e.*, the amount of β-oxidation products present in the mixture.

EXPERIMENTAL.

Variations in the Alkalinity.

Experiment I. Action of Hydrogen Peroxide on Potassium Butyrate in the Presence of Variable Concentrations of Alkali.—4.4 gm. of butyric acid were weighed out in each of five flasks. They were all neutralized with potassium hydroxide. Additional potassium hydroxide was added as follows: to (1) none; to (2) 1.4 gm. or 0.5 molecule, to (3) 2.8 gm. or 1 molecule; to (4) 5.6 gm. or 2 molecules; to (5) 11.2 gm. or 4 molecules. They were all diluted to 387 cc., and 113 cc. of 3 per cent hydrogen peroxide were added (total volume 500 cc.), and the mixtures were allowed to stand at room temperature for 1 month. Even at the end of this time they all gave positive tests for hydrogen peroxide. 100 cc. of each of these solutions were distilled at ordinary pressure by heating the distilling flask with a ceresin bath, at a bath temperature of not over 160°. The distillate of (1) had a strong odor resembling acrolein or crotonic aldehyde. This odor diminished in the others and was absent in (5). These distillates were titrated for acetone by the Lieben iodoform method. From the amount of iodine solution that was consumed in each case the

amount of acetone that could have been present in each experiment was as follows: (1) 0.293 gm., (2) 0.0973 gm., (3) 0.0567 gm., (4) 0.0655 gm., (5) 0.0675 gm. In verifying these results the solutions were used up. In every case solid iodoform separated out and the odor was unmistakable.

Since in this series of experiments no effort was made to eliminate other compounds that form iodoform or that absorb iodine, another series was run. The same amounts of butyric acid and hydrogen peroxide were used. Each was diluted to a volume of 500 cc. The amounts of potassium hydroxide used were as follows: (1) no excess; (2) 0.125 molecule or 0.35 gm. in excess; (3) 0.25 molecule or 0.7 gm. excess; (4) 3.8 molecule or 1.05 gm. excess; (5) 0.5 molecule or 1.4 gm. excess; (6) 0.625 molecule or 1.75 gm. excess; (7) 0.75 molecule or 2.1 gm. excess; (8) 0.875 molecule or 2.45 gm. excess; (9) 1 molecule or 2.80 gm. excess; (10) 1.5 molecules or 4.2 gm. excess; (11) 2 molecules or 5.60 gm. excess; (12) 3 molecules or 8.4 gm. excess. These were distilled off as above after standing at room temperature 40 days. The amount of iodine consumed corresponded in (1) to 0.354 gm. of acetone, in (2) to 0.0764 gm. of acetone, in (3) to 0.0484 gm. of acetone, and in the remainder fluctuated a little but was about the same as in (3). Since in test experiments it had been found that when an acetaldehyde solution of known iodoform titer was heated with excess silver oxide in the presence of potassium hydroxide, in pressure flasks for 2 or 3 hours, it no longer absorbed iodine when titrated by the customary iodoform method, the above solutions were submitted to the same treatment. After the treatment was complete the silver and excess silver oxide were filtered out, the precipitate and filter were washed, and the filtrate and washings again distilled, because the silver salts interfered with the titrations. The acetone titrations obtained with these distillates corresponded in (1) to 0.161 to 0.175 gm. of acetone; in (2) to 0.030 gm. of acetone, and in the remainder of the experiments the values fluctuated between 0.030 and 0.040 gm. of acetone in each case, when calculated for the entire solution.

The results of both sets of experiments show that the yield of acetone was greatest in the flasks containing the least alkali and declined rapidly in the flasks containing more alkali.

Experiment II. The Effect of the Total Amount of Alkali on the Yield of Acetone in the Oxidation of Butyric Acid by Hydrogen Peroxide.—In some earlier experiments on the oxidation of glucose with potassium permanganate[4] in the presence of variable concentrations of alkali, it was found that there was a sharp change to a maximum yield of oxalic acid when the total amount of alkali present was sufficient to neutralize completely all of the acids produced by the oxidation. Since variations in the alkali concentrations of the body tissues must of necessity be small, but since the total available neutralizing power may easily vary in a much larger sense, it seemed desirable to repeat the oxidation of potassium butyrate under conditions in which the initial concentration of the alkali in each experiment is the same while the total amount varies. In each experiment the potassium salt of 4.4 gm. of butyric acid and 113 cc. of 3 per cent hydrogen peroxide were used. The experiments and amounts of acetone obtained were as follows:

	Total volume 0.01 N alkali.	Acetone.
	cc.	*gm.*
(1)	200	0.163
(2)	400	0.075
(3)	600	0.050
(4)	800	0.042
(5)	1,000	0.050
(6)	1,600	0.043
(7)	2,000	0.046
(8)	4,000	0.045

The above results are strictly comparable with the results obtained in Experiment I in which the concentration of the alkali was varied. There is no question, therefore, but that the amount of alkali available is a fundamental influence on the amount of acetone found in these experiments.

Critical Experiments.

It was thought that perhaps the acetone formed in the more alkaline flasks in Experiments I and II was being (*a*) condensed by the alkali and (*b*) being oxidized by the peroxide. (*a*) was tested first.

[4] Witzemann, E. J., *J. Am. Chem. Soc.*, 1916, xxxviii, 150.

(*a*) *Action of Alkali on Acetone.*—50 cc. of a titrated acetone solution containing 0.05 gm. of acetone were mixed with 20 cc. of 20 per cent potassium hydroxide solution and 80 cc. of water, making the solution 0.47 N alkali, and distilled. The distillate showed 0.05 gm. of acetone. 10 cc. of this 0.1 per cent acetone solution were added to each of three flasks containing 200 cc. of water, and 10, 20, and 30 cc. respectively, of 20 per cent potassium hydroxide were added. After standing 3 days at room temperature, all showed an identical titer of 1.0 gm. of acetone per liter for the acetone solution used.

These experiments showed that acetone is not condensed by alkali under the conditions used in these experiments.

Portions of this acetone solution were also subjected to the treatment with alkaline silver oxide. In all cases some of the acetone was lost, showing thus that the amount of acetone surviving this treatment will be smaller than the amount originally present.

In view of all the experimental work of other authors on the iodoform method for determining acetone and the experiments described in the preceding paragraphs, it is probable that the data obtained by direct titration of the distillates in oxidation experiments like I and II give a result that is too high when calculated entirely to acetone. Moreover, the titrations for acetone are probably too low after the treatment with silver oxide and alkali. But assuming that both series of results show the relative amounts of acetone present, it is clear that the amount of acetone present is larger the closer neutrality is approached. These experiments outwardly definitely verify the idea that a larger proportion of acetone bodies is formed in the more acid conditions.

This conclusion is modified by special experiments designed to test its truth, which are described under (*b*).

(*b*) *The Action of Hydrogen Peroxide on Acetone in Weakly Alkaline Solutions.*—In some experiments on the oxidation of acetone with potassium permanganate in the presence of variable amounts of alkali[5] it was found that at low concentrations of alkali the acetone mixture (2 gm. of acetone in 1 liter of solution)

[5] Witzemann, *J. Am. Chem. Soc.*, 1917, xxxix, 2657.

came to equilibrium with unchanged acetone and oxidation could not be continued.[6]

It was of interest to know whether the same thing occurs when hydrogen peroxide is used. It was found that although hydrogen peroxide does not act so vigorously as permanganate under these conditions, it nevertheless qualitatively gives the same result.

50 cc. of an acetone solution containing about 2 gm. in 50 cc. were added to 900 cc. of a solution containing enough alkali for 1 liter, having the following concentrations: (1) blank, no alkali; (2) no alkali; (3) 0.01 N alkali; (4) 0.02 N alkali; (5) 0.03 N alkali; (6) 0.05 N alkali; (7) 0.10 N alkali. After standing 5 days at room temperature 5 cc. were pipetted off and titrated for acetone. Since the titration values were practically identical it was again shown that acetone is not condensed by these concentrations of alkali. 50 cc. of hydrogen peroxide (3.07 per cent) were then added to each flask except (1) to which 50 cc. of water were added. After remaining 13 days in a dark cupboard, hydrogen peroxide was tested for by the chromic acid-ether test. Only (2) showed the presence of hydrogen peroxide in this way.

5 cc. of each of the above solutions were pipetted off and placed in a flask with about 30 cc. of water. A pinch of manganese dioxide was added to decompose the hydrogen peroxide. Only two showed any evolution of oxygen. The mixtures were carefully filtered, the filters washed, and the filtrates titrated for acetone as usual.

In each experiment there were originally 2.11 gm. of acetone. The titrations showed a loss of 0.096 gm. or 4.8 per cent in (1); 0.096 gm. or 4.8 per cent in (2); 0.28 gm. or 13.2 per cent in (3); 0.30 gm. or 14.2 per cent in (4); 0.39 gm. or 18.5 per cent in (5); 0.46 gm. or 21.8 per cent in (6); 0.47 gm. or 22.2 per cent in (7). A careful restandardization of the iodine and thiosulfate solution showed that their titer was unchanged and the loss in (1) and (2) was presumably due to other causes than errors in titration. That this error was incurred during the treatment with manganese dioxide and the subsequent filtration was shown in another series of experiments in which the hydrogen peroxide was

[6] Sachs F., *Ber. chem. Ges.*, 1901, xxxiv, 497.

all decomposed without any treatment with manganese dioxide and in which the acetone content was found to be practically unchanged. If these solutions were then put through the manganese dioxide treatment the acetone loss was about 5 per cent. Thus if 4.8 per cent is subtracted from the above values, since presumably the evaporation of acetone was about the same in all cases, the values remaining would more truly represent the amount of acetone destroyed by hydrogen peroxide.

	per cent		per cent
(1)	0.0	(4)	9.4
(2)	0.0	(5)	13.5
(3)	8.4	(6)	17.0
		(7)	17.4

If 0.47 gm. of acetone in (7) was oxidized to one molecule of acetic acid plus one molecule of formic acid, then three atoms of oxygen, or 0.39 gm., or 0.83 gm. of hydrogen peroxide, would be required. Since 1.53 gm. of hydrogen peroxide were added in the above experiments it is clear that there was enough hydrogen peroxide present to oxidize nearly twice the maximum amount of acetone oxidized to acetic and formic acids. If the sole products were acetic and carbonic acids, 1.11 gm. of hydrogen peroxide would be required. It is thus clear that the amount of peroxide added could have produced the observed amount of oxidation. The nature of the products was not determined.

The above experiments were repeated a number of times under a variety of conditions designed to prevent loss of acetone by evaporation during the oxidation. This result was not realized perfectly in any complete series. Although the quantitative results varied somewhat in each case the general sense of the results was always the same. It would not be profitable to describe the individual results since the nature of the variations causing the isolated abnormal results is not known with certainty.

The above experiments show that acetone is more and more easily oxidized by hydrogen peroxide with increasing alkali concentrations. That all of the hydrogen peroxide is not used in oxidation of acetone is shown by the fact that it sometimes disappears from the solution (kept in the dark) without producing any noticeable oxidation. Thus perhaps the result in any experi-

ment is the resultant between the velocity of catalytic decomposition of the hydrogen peroxide and the velocity of oxidation of acetone by it. It will not be advisable at present to discuss the mechanism of the processes involved here.[7] It is sufficient to note that acetone in the presence of hydrogen peroxide and varying amounts of alkali comes to equilibrium with the system with an increasing stability of acetone with decreasing alkali concentration.

May acetone be oxidized by air? In connection with the above experiments the question arises as to whether all or part of the oxidation of acetone observed may not be due to the oxygen of the air. In order to answer this question about 1 gm. of acetone was made up to 500 cc. with enough alkali in each case to make the concentration as follows: (1) no alkali; (2) 0.01 N alkali; (3) 0.05 N alkali; (4) 0.10 N alkali; (5) 0.20 N alkali. 5 cc. of the mixtures were titrated for the acetone as usual. 1.04 gm. was the average acetone content. The flasks were stoppered tightly with rubber stoppers and were agitated vigorously at least once each day. After 12 days the average acetone content was 1.03 gm. After 1 month the average acetone content was found to be 1.03 gm. These experiments show that if atmospheric oxygen cooperates with hydrogen peroxide in the oxidation of acetone it does so at some lower stage in the oxidation than acetone.

These results of the experiments described under (*b*) prove that acetone formed in experiments like I and II is liable to be partly oxidized under the conditions of the experiment and that the amount of acetone found is not necessarily all that was formed.

Rigidly interpreted this result would indicate that we no longer have any conclusive evidence that a greater proportion of acetone is formed in the less alkaline flasks in Experiments I and II. In fact, a closer examination of the results in Experiments I, II, and (*b*) shows that in each case the amount of acetone oxidized in the corresponding experiment in (*b*) by less than half as much hydrogen peroxide is greater than the amount of acetone observed in the corresponding experiment in I and II. This result, however, does not in any sense invalidate the sug-

[7] The interrelation of these two processes is being investigated experimentally.

gestion that a greater proportion of acetone bodies is formed when butyric acid is oxidized in less alkaline conditions. It only shows that the value of this suggestion cannot be established in this way. The one positive result that comes from these experiments is the confirmation of the suggestion that acetone, and presumably the acetone bodies, is oxidized with greater difficulty the less alkaline the solution becomes when all other conditions are identical.

Variation of Glucose.

Experiments to Determine the Effect of the Presence of Glucose on the Yield of Acetone from a Constant Amount of Butyric Acid, Hydrogen Peroxide, and Alkali.—As was stated in the introduction, the presence of glucose undergoing oxidation has the effect of diminishing the ketosis under discussion. Can this effect be produced *in vitro*, and if so under what conditions? The following two series of experiments were done in order to get some experimental light on this subject.

Experiment III.—4.4 gm. of butyric acid in the form of the potassium salt in a total volume of 600 cc., containing enough potassium hydroxide to be 0.01 N at the beginning, and 113 cc. of 3 per cent hydrogen peroxide also contained glucose as follows: (1) no glucose; (2) 0.09 gm.; (3) 0.27 gm.; (4) 0.45 gm.; (5) 0.90 gm.; (6) 1.80 gm. After standing 1 month in the dark (1) was free from hydrogen peroxide, (2), (3), and (4) showed traces, and (5) and (6) gave strong tests for hydrogen peroxide. These solutions were analyzed for acetone in the usual way and even after treatment with alkaline silver oxide showed a gradually but markedly increasing acetone content with increasing glucose content. It was immediately suspected that the available alkali had been progressively consumed. Titrations showed that only (1) and (2) were still alkaline. The others were all distinctly acid in reaction. This result, although not exactly what was desired, is extremely interesting when taken in conjunction with the next series of experiments and will be emphasized later on.

Experiment IV.—The presence of variable amounts of glucose caused the amount of available alkali in turn to vary. In order to eliminate this additional variable as much as possible the experiments were again set up in the same way and small, known

portions were removed each day at first and then every few days, and titrated with 0.1 N sulfuric acid. The calculated amount of alkali was added each time to bring the alkali concentration up to its original value (0.01 N). The total volume of potassium hydroxide, 0.84 N, added to each flask in this way was as follows: (1) 10.97 cc.; (2) 11.97 cc.; (3) 13.44 cc.; (4) 17.87 cc.; (5) 21.76 cc.; (6) 26.02 cc. The amount of alkali thus consumed gives a relative index of the amount of acid production that occurs in these oxidations. Since the amount of potassium butyrate used was constant it is easy to ascribe the variable alkali consumption to the glucose oxidation. Owing to the fact that the mixture was constantly alkaline only a small amount of acetone was found even in Flask 1. In (2) still less was formed and in the remainder the amount was quite small and nearly constant as far as the data showed. In these experiments the amount of iodine consumed by the distillate before and after treatment with alkaline silver oxide was nearly identical. This is in marked contrast with the first series of experiments in which the iodine consumed by the untreated distillates increased with the sugar content and clearly indicates a marked difference in the final state of the compounds in the two solutions. The latter series of experiments gave the expected results and when taken in conjunction with the first series demonstrates clearly in another way the marked and fundamental importance of an adequate supply of alkali in such oxidations.

Experiment V. Effect of the Presence of Glucose on the Yield of Acetone from a Constant Amount of Butyric Acid and Hydrogen Peroxide in the Presence of So Called Regulator Mixtures.—There seems to be a feeling that the source of alkalinity influences oxidation. Thus in order to put the above experiments on a basis that would be more readily acknowledged to resemble biological conditions they were repeated in the presence of so called regulators. Preliminary experiments showed that calcium carbonate, calcium hydroxide, and sodium carbonate are not available for these experiments since they so rapidly catalyze the decomposition of hydrogen peroxide. It was therefore necessary to use the phosphate mixture. In these experiments it was desired to use enough hydrogen peroxide to oxidize both the butyric acid and glucose used to carbon dioxide. Since acetone itself is gradually oxidized by peroxide the oxidation was stopped after a time before the peroxide had all been reduced.

The experiments were conducted as follows: The amounts of K_2HPO_4 and KH_2PO_4 given in the first two columns of the table were dissolved in 50 cc. of water, the solution was cooled, and 0.25 gm. of butyric acid as the potassium salt (5 cc. of a 5 per cent solution) was added. 0.25 gm. of glucose (0.5 molecule) was also added to the second flask. 50 cc. of 3 per cent hydrogen peroxide were then added to both. In Experiments 7 and 8 the salts were dissolved in 150 cc. of water. After standing at room temperature for 24 hrs. stoppered with a rubber stopper, the entire mixture was transferred to the distilling flask and after adding some precipitated calcium carbonate to facilitate the decomposition of excess hydrogen peroxide and some broken porcelain to facilitate boiling, the acetone was distilled as usual. The results of such experiments should be expected to vary somewhat but in general they tend to run parallel with the results obtained above, using potassium hydroxide. In Experiments 1 to 6 more acetone is obtained at first in the presence of glucose than in its absence but as the available alkali is increased (K_2HPO_4) this advantage diminishes and in the later experiments the acetone production is about equal in the presence and absence of glucose. The use of larger amounts of K_2HPO_4 without changing the proportions, as in Experiments 7 and 8, likewise tends to equalize the acetone production in the presence and absence of glucose.

Experiment No.	K_2HPO_4	KH_2PO_4	With glucose.		Without glucose.	
			Total acetone.	Acetone after Ag_2O+KOH.	Total acetone.	Acetone after Ag_2O+KOH.
	gm.	gm.	gm.	gm.	gm.	gm.
1	5.35	6.92	0.096	0.0264	0.057	0.0135
2	6.92	5.35	0.089	0.0154	0.040	None.
3	6.92	5.35	0.081	0.0198	0.058	0.011
4	7.92	4.35	0.074	0.0133	0.112*	0.024
5	7.92	4.35	0.077	0.0197	0.075	0.019
6	8.92	3.35	0.075	0.0202	0.087	0.022
7	20.76	16.05	0.081	0.0248	0.075	0.026
8	20.76	16.05	0.086	0.0264	0.085	0.025

* Boiled dry in distillation.

Discussion of Experiments III, IV, and V.

Owing to the well known relationships of glucose oxidation to the formation of the acetone bodies in fasting and diabetes, it was important to observe the effect of the presence of variable amounts of glucose on the yield of acetone from a constant amount of butyric acid, hydrogen peroxide, and alkali. It was found in Experiment III that the products of glucose oxidation caused a progressive diminution in the alkalinity and accordingly an increasing production of acetone with increasing amounts of glucose. That is, when an inadequate supply of alkali is present in the presence of sufficient oxidizing agent and increasing quantities of glucose, there is an increased production of acetone. In this series glucose not only does not act antiketogenically but seems to favor ketogenesis. In Experiment IV in which the alkali supply was maintained the acetone production was at a minimum in all but those flasks in which there was little or no sugar present. In Experiment III glucose favored ketogenesis; in Experiment IV it was antiketogenic. The reversal in its effect was due to the control of the alkalinity. Experiment IV gives *in vitro* confirmation for the idea that the sugar spares[8] the oxidation of fats in the presence of adequate alkali supplies. The mechanism by which this may be effected is suggested by the result of Experiment VI.

[8] The word "spares" in this paper is apparently not used in the same sense as it has previously been used in this connection by other writers. It is used here in a figurative sense as explained below. According to the coexistence principle (see Ostwald, *Lehrb. allg. Chem.*, 1896–1902, ii, 244, 307) two reactions taking place in the same medium simultaneously proceed independently. When, however, both reactions are taking place at the expense of a single common reagent (in this case the oxidizing agent), not present in enormous excess, the velocity of each reaction from moment to moment depends on the velocity of the other reaction during the preceding period. If one reaction is using oxygen very rapidly the amount available for the other is more rapidly diminished. If one reaction is markedly stimulated with respect to the other for any given set of conditions, the compound being oxidized by the slower reaction may be said to be spared. Thus sparing in this sense is a chemical conception and contains no hint of a vitalistic point of view. This conception of sparing is involved in Experiments III, IV, V, and VI. More definite experiments on this subject are already partly completed.

Experiment V was a combination of all the preceding experiments in which mixtures of mono- and dibasic potassium phosphate were used instead of alkali. Since mixtures of these salts constitute an important part of the mechanism by which the reaction of the body fluids is regulated, it was thought that results obtained with such systems would be more readily acceptable. The general sense of the results confirms all that had been determined by the previous experiments, both as to the effect of available alkali on butyric acid oxidation and on the effect of glucose on this oxidation, without giving rise to any new conclusions.

Variation of Acidity.

The preceding experiments have shown that the less alkaline the system the greater the amount of acetone that was recovered from the experiment. It was of interest to know whether the amount of acetone that can be recovered continues to increase with increasing acidity. A few simple experiments were done to test this possibility, since there are theoretical reasons why it should be expected to be so.

Experiment VI.—The experiments were set up as follows: Each flask contained 50 cc. of 3 per cent hydrogen peroxide, and potassium butyrate and butyric acid (measured as standardized solutions) as follows: (1) 0.25 gm. of potassium butyrate; (2) 0.20 gm. of potassium butyrate and 0.05 gm. of butyric acid; (3) 0.15 gm. of potassium butyrate and 0.10 gm. of butyric acid; (4) 0.25 gm. of butyric acid. The total volume in each case was 100 cc. After standing at room temperature 42 hours, excess powdered manganese dioxide was added to decompose the large amount of unchanged hydrogen peroxide. When this decomposition, which was carried out in the distilling flask connected as for distillation, was complete, the entire solution was distilled and titrated for acetone as usual. The iodine consumed corresponded to about 0.001 gm. of acetone in each case. The total variation for the series did not exceed 10 per cent of the amount of acetone found. No solid iodoform was separated in these experiments but the odor of iodoform was unmistakable.

The results of this experiment indicate that the oxidation is so slow under these conditions that a difference in the amount of

acetone obtained is not clearly demonstrable after standing 42 hours, or else that there is no demonstrable difference. Since such experiments in the light of the above critical experiments could not give a satisfactory answer to the question as to whether there is an increase in the ratio of acetone formed, these experiments in acid conditions were not extended.

Similar experiments in which four molecules of sulfuric and hydrochloric acids were used for each molecule of potassium butyrate gave no striking increase in the yield of acetone, but with phosphoric acid an unexpectedly marked increase in acetone was observed.

This observation when taken in conjunction with the large acetone yields obtained with the phosphate mixtures in Experiment V opened up this problem from another angle. The experiments developed in this connection will be described in another paper.

Oxidation of Acetone and Glucose.

Experiment VII. Simultaneous Oxidation of Acetone and Glucose with Potassium Permanganate in the Presence of Varying Concentrations of Alkali.—Hydrogen peroxide presents one grave disadvantage as a quantitative oxidizing agent in alkaline systems owing to the fact that it is catalytically decomposed by alkali. Since the velocity of this decomposition varies with the composition of the system no quantitative results on the amount of oxygen consumed can be obtained in this way. Therefore in any study of the sparing effect of one metabolite upon another some other oxidizing agent in which this spontaneous decomposition is absent or negligible must be used. Since acetone may be regarded as one end-member of the β-oxidation of butyric acid which is difficult to oxidize, it was of interest to learn to what extent glucose spares the oxidation of acetone in systems containing an equal amount of both but a variable amount of alkali. Ten experiments were done as follows: 0.5 gm. of acetone, 0.5 gm. of glucose, and 4.0 gm. of potassium permanganate (insufficient for complete oxidation of either the glucose or acetone alone) were placed in 500 cc. of a solution containing alkali as follows:

		Acetone.	
		gm.	*per cent*
(1)	No alkali nor $KMnO_4$ (blank)	0.547	100.0
(2)	" "	0.403	73.0
(3)	0.6 gm. KOH (0.021 N)	0.326	59.6
(4)	1.21 " " (0.043 ")	0.261	47.7
(5)	1.82 " " (0.064 ")	0.237	43.3
(6)	2.43 " " (0.087 ")	0.219	40.0
(7)	3.03 " " (0.108 ")	0.218	39.8
(8)	6.06 " " (0.216 ")	0.194	35.4
(9)	19.44 " " (0.694 ")	0.200	36.5
(10)	38.88 " " (1.388 ")	0.185	33.8

After reduction was complete, *i.e.* after all the permanganate was reduced to manganese dioxide, the acetone content was determined in the usual way. The results are given in the last column. The data show that two and one half times as much acetone was oxidized in Flask 10 as in Flask 2 and that the transition is gradual. From these experiments it may be concluded that the capacity of glucose to spare acetone from oxidation diminishes with increasing alkalinity, or conversely the capacity of acetone to spare glucose diminishes with decreasing alkalinity. Or in physical chemical terms, the velocity of oxidation of acetone increases rapidly with increasing alkalinity even in the presence of glucose.

But not only does the velocity of oxidation of acetone with respect to glucose in the same system vary but also the velocity of permanganate reduction as a whole varies markedly from one experiment to the other. The velocity increases with rising alkalinity. In the more alkaline experiments it becomes so rapid as to be apparently instantaneous. In the case of Flask 2 several days were required for complete reduction.[9]

This experiment gives further illustration of the complexity of the processes involved in oxidation.

DISCUSSION.

Experiments I and II indicate that when butyric acid is oxidized by means of hydrogen peroxide in dilute solution in the presence of variable amounts of alkali the absolute quantity of

[9] The above is simply a preliminary account of one result in a more comprehensive study of this question that is under way in this laboratory.

acetone recoverable is greatest when the reaction of the system is neutral to faintly acid. The preliminary data mentioned in connection with Experiment VI indicate that the nature of this acidity has some influence on the yield of acetone. The important question as to the exact conditions that promote a maximum yield of acetone will not be discussed until the effect of other variables has been more fully studied.

The yield of acetone falls as the alkalinity increases notwithstanding an increase in the velocity of the oxidation of butyric acid. This decrease of the acetone yield is explainable in part if not wholly by the fact that acetone itself becomes increasingly susceptible to oxidation with the increasing alkalinity, but it was not proved that this phenomenon accounts for the entire decrease of the acetone yield. The original question as to whether the point of primary oxidative attack may be shifted by changing the reactions of the medium remains unanswered.

In studying the effect of the presence of glucose it was found that oxidizing glucose may change the reaction of the medium and so influence the acetone yield. But we have found nothing to indicate that the oxidation of glucose simultaneously with butyric acid influences the nature of the products formed from the latter.

On the other hand, Experiments III, IV, and V demonstrate how glucose may "spare" butyric acid in a manner which affords an interesting chemical analogy to the so called "sparing effect" of carbohydrate in the body.

Experiment VII gives a graphic illustration of how this "sparing" effect too may vary with changes in the reaction.

It will not be advisable to discuss the above results at any greater length but a few more general statements should be made. In any such complicated process as the oxidation of butyric acid or acetone there are many possible courses of reaction through many intermediate stages. The velocity of each of these reactions is influenced by minute changes in conditions. Some are more sensitive than others. In order to understand thoroughly the net result in such a case it would be necessary to know the constants for each set of conditions for each reaction. But since in biological conditions these reactions are carried out in heterogeneous systems devised to prevent the attainment of complete

equilibrium in life, such constants worked out in homogeneous systems *in vitro* might only have qualitative or no significance in the biological conditions. Nevertheless it seems certain that the experiments described here represent a constructive step toward a solution of this problem.

AMMONIA EXCRETION AS INFLUENCED BY THE INGESTION OF ALKALIES.

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So much experimental work has been carried out on the influence of alkali ingestion on urinary ammonia that further investigations along this line would seem superfluous.

The great reduction in the ammonia output produced by the administration of relatively small doses (10 to 20 gm. per day) of sodium bicarbonate has led to the general acceptance of the view that the sole purpose of urinary ammonia is for the neutralization of acids formed during the metabolic processes.

On the other hand continued administration of alkali for many days has in no case resulted in the production of a urine free from ammonia,[1] a finding which has led to speculations by several investigators regarding the source of the residual fraction.[2-4] An examination of the reports of the numerous series of experiments dealing with the effect of alkali administration will reveal the fact that similar doses of sodium bicarbonate will produce quantitatively different effects as regards urinary ammonia when administered to a series of subjects living under essentially similar dietary conditions.

Diminished power to reduce urinary ammonia might, under these conditions, be interpreted to indicate either the presence of an increased amount of acid radicals requiring neutralization, or

[1] Janney, N., *Z. physiol. Chem.*, 1911–12, lxxvi, 99, contains an extensive bibliography on this subject.

[2] Magnus-Levy, A., in von Noorden C., Handf. Path. Stoffwechsels, Berlin, 1906, i, 111.

[3] Neubauer, O., *Verhandl. deutsch. Cong. inn. Med.*, 1910, xxvii, 566; *Arch. exp. Path. u. Pharmakol.*, 1901, xlvi, 133.

[4] Camerer, W., Jr., *Z. Biol.*, 1902, xliii, 39.

the excretion of the alkali, either by the intestine or kidney, before its neutralizing effect has been exerted. Similarly the occurrence of an exaggerated effect might be attributed to a low concentration of acid radicals or to the abnormally slow excretion of the drug.

The inefficiency of the nephritic kidney in the task of eliminating sodium salts has long been recognized. It therefore occurred to us that in the nephritic, urines of minimum ammonia content might be obtained by means of relatively small doses of alkali.

As will be seen by an examination of the results obtained in Experiments I, II, and III, our expectations were abundantly fulfilled as we have been able in two of these patients to obtain urines absolutely free from even traces of ammonia, and in some cases to prolong this condition for 1 or 2 days after the administration of alkali had been discontinued.

Subjects 2 and 3 ate during the entire experimental period a low protein diet containing many vegetables. The same food was also taken by Subject 1 during the first part of the experimental period. During the last 15 days, however, he was given food containing as much protein as he could be induced to take, but as will be seen even on this more acid diet an ammonia-free urine was easily obtained for a period of 3 days.

The figures of experiments I to III give the results obtained by the intermittent administration of sodium bicarbonate. This method of dosage was adopted by us as being more feasible for long continued experiments in order to avoid the nausea which frequently follows the uninterrupted use of alkali for a considerable number of days. The sodium bicarbonate was given in 5 gm. doses at 7.00 a.m. and at 7.00 p.m.; where more than 10 gm. per day were used the other portions were administered at 11.00 a.m. or at 2.00 p.m.

To serve as controls on the results obtained with these three cases of nephritis we have carried out experiments with two normal subjects who consumed the same alkaline low protein diet as that taken by the nephritics, and one case of catarrhal jaundice who was given the higher protein diet taken by Subject 1 during the latter part of the experimental period.

The results of these experiments require little comment, as they merely serve to confirm the findings of previous investi-

EXPERIMENT I.

Subject 1.—Mr. B. 38 years old. Weight 79 kilos. Nephritis. Much edema. Urine contains a large trace of albumin and many granular and hyaline casts.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Sodium bicarbonate taken.
1917	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Nov. 27–28	540	1.13	0.180	5.1	0
" 28–29	400	1.10	0.140	5.6	10
" 29–30	400	1.20	0.121	4.8	15
" 30–Dec. 1	350	1.07	0.042	5.0	15
Dec. 1–2	375	1.21	0.032	5.5	15
" 2–3	370	1.22	0.035	5.9	0
" 3–4	380	1.18	0.038	5.4	0
" 4–5	400	1.14	0.040	5.7	15
" 5–6	540	1.18	0.011	5.9	15
" 6–7	640	1.22	0.000	7.3	15
" 7–8	410	1.18	0.000	4.87	0
" 8–9	550	1.08	0.001	4.89	0
" 9–10	400	1.18	0.064	4.83	0
" 10–11	400	1.20	0.118	4.52	5
" 11–12	360	1.11	0.092	4.60	10
" 12–13	400	1.18	0.100	4.04	15
" 13–14	360	1.18	0.091	5.14	10
" 14–15	320	1.16	0.100	4.8	10
" 15–16	350	1.10	0.102	4.9	10
" 16–17	340	1.09	0.057	5.4	10
" 17–18	320	1.07	0.038	4.9	10
" 18–19	350	1.15	0.018	4.0	15
" 19–20	540	1.25	0.000	5.4	15
" 20–21	660	1.10	0.004	4.1	0
" 21–22	540	1.19	0.036	6.3	0
" 22–23	380	1.25	0.040		0
" 23–24	280	1.01	0.035		0
" 24–25	320	1.00	0.032		0
" 25–26	310	1.24	0.000		10
" 26–27	350	1.01	0.042		0
" 27–28	470	1.00	0.042		0
" 28–29	600	1.01	0.046		0
" 29–30	680	1.00	0.082		0
" 30–31	780	1.20	0.063		0

EXPERIMENT I—*Concluded.*

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Sodium bicarbonate taken.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Jan. 1–2	550	1.08	0.069	9.13	0
" 2–3	680	1.18	0.035	10.33	10
" 3–4	900	1.29	0.051	10.71	10
" 4–5	1,060	1.37	0.060	10.60	10
" 5–6	1,007	1.28	0.030	9.83	10
" 6–7	1,050	1.27	0.032	9.53	10
" 7–8	1,020	1.18	0.040	10.51	10
" 8–9	1,370	1.36	0.000	11.7	15
" 9–10	1,120	1.30	0.000	10.81	0
" 10–11	1,000	1.17	0.000	9.92	0
" 11–12	930	1.20	0.026	8.72	0
" 12–13	1,250	1.27	0.112	8.35	0
" 13–14	1,380	1.27	0.165	11.59	

EXPERIMENT II.

Subject 2.—Mr. T. 27 years old. Weight 72 kilos. Nephritis. Marked edema, urine contains a large trace of albumin, many casts, and a few red blood cells.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Sodium bicarbonate taken.
1917	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Nov. 24–25	1,420	1.20	0.24	7.3	0
" 25–26	710	1.10	0.19		10
" 26–27	830	1.11	0.080	5.7	15
" 27–28	1,070	1.10	0.050	6.09	15
" 28–29	1,100	1.11	0.035	7.15	15
" 29–30	1,600	1.11	—	—	0
" 30–Dec. 1	1,570	1.11	—	—	0
Dec. 1–2	1,650	1.20	0.160	6.55	0
" 2–3	1,250	1.10	0.110	6.51	0
" 3–4	1,100	1.12	0.100	5.94	0
" 4–5	740	1.02	0.040	5.37	15
" 5–6	930	1.10	0.011	5.38	15
" 6–7	1,800	1.21	0.000	8.10	15
" 7–8	1,600	1.04	0.000	5.28	0
" 8–9	1,600	1.04	0.002	5.92	0
" 9–10	740	1.00	0.007	5.24	0
" 10–11	1,320	1.00	0.042	5.78	0

EXPERIMENT III.

Subject 3.—Mr. P. 58 years old. Weight 52 kilos. Nephritis, chronic interstitial. Urine shows slight trace of albumin and a few hyaline casts.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Sodium bicarbonate taken.
1917	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Nov. 23–24	600	0.84	0.26	4.6	0
" 24–25	670	0.80	0.22	4.1	0
" 25–26	710	0.80	0.23	5.2	10
" 26–27	680	0.91	0.19	4.7	15
" 27–28	850	1.00	0.16	5.7	15
" 28–29	840	1.00	0.14	5.4	15
" 29–30	1,020	0.90	0.058	5.4	15
" 30–Dec. 1	1,220	0.90	0.043	4.9	15
Dec. 1–2	1,500	0.81	0.010	4.6	15
" 2–3	1,380	1.00	0.002	5.7	0

All urines were preserved with thymol and chloroform. The ammonia determinations were made by means of "Permutit" according to the method of Folin and Bell.[5] In the tabulated results we have also included the figures obtained for urinary creatinine. It has for several years been our custom in this laboratory to determine creatinine on all 24 hour quantities of urine collected during the course of metabolism experiments, and the results are here included on the tables merely to allow the reader to form his own judgment as to the completeness of the collections.

gators that it is impossible in the normal subject to obtain ammonia-free urine by the long continued administration of alkalies.

In the two normal subjects bicarbonate ingestion was continued until severe headache and nausea resulted. In spite of the fact that in Subject 4 the urine remained strongly alkaline for 10, and in the case of Subject 5 for 15 consecutive days, it will be noted that considerable amounts of ammonia were still present in the urine at the end of the experiment.

We have interpreted the essentially different results obtained on the administration of alkali to nephritics from those obtained by the use of a similar dosage on normal individuals as being due to the retention of the sodium bicarbonate for a longer time by these patients, thus allowing the drug more time to exert its neutralizing effects.

In the investigations on alkali ingestion so far reported but little effort has been made to measure even approximately the

[5] Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

EXPERIMENT IV.

Subject 4.—Normal female. 39 years old. Weight 90 kilos.

Date.	24 hr. volume.	NH_3	Total N.	Sodium bicarbonate taken.	Reaction of urine to litmus.
1918	*cc.*	*gm.*	*gm.*	*gm.*	
Feb. 7–8	1,080	0.231	8.10	0	Acid.
" 8–9	1,260	0.150	5.90	10	Alkaline.
" 9–10	2,180	0.170	7.5	15	"
" 10–11	1,750	0.087	5.2	15	"
" 11–12	1,600	0.035	6.1	15	"
" 12–13	1,640	0.186	5.9	0	"
" 13–14	1,130	0.210	4.5	0	Acid.
" 14–15	1,260	0.200	—	8	Alkaline.
" 15–16	—	—	—	10	"
" 16–17	1,280	0.150	4.9	10	"
" 17–18	—	—	—	10	"
" 18–19	1,750	0.115	6.0	10	"
" 19–20	—	—	—	10	"
" 20–21	1,740	0.069	5.3	10	"
" 21–22	—	—	—	10	"
" 22–23	1,340	0.070	7.8	10	"
" 24–25	2,490	0.090	6.8	10	"

amount of unchanged alkali excreted. From experiments IV to VI it appears possible that the different effects produced by the same dosage of alkali on the urinary ammonia of different individuals might be due in most cases to the relative activity of the different kidneys and the resulting removal of the reactive substance at diverse rates of speed.

In our later experiments we have therefore sought to determine not only the changes produced in ammonia excretion by alkaline therapy but have also attempted to measure approximately the amount of ingested alkali appearing in the urine.

The excretion of sodium bicarbonate was followed by determinations of total ash and total carbon dioxide in the urine. The ash determinations were carried out in platinum dishes which were heated in a muffle furnace, due care being taken to avoid loss due to overheating. Total carbon dioxide was determined by the method of Van Slyke.[6] Routine qualitative tests for sodium bicarbonate were made on the stools but were invariably negative.

In order to avoid the fluctuations in the urinary carbon dioxide and ash referable to changes in diet, all the subjects were required to eat a weighed

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

EXPERIMENT V.

Subject 5.—Normal female. 24 years old. Weight 53 kilos.

Date.	24 hr. volume.	NH_3	Total N.	Sodium bicarbonate taken.	Reaction of urine to litmus.
1918	*cc.*	*gm.*	*gm.*	*gm.*	
Feb. 7–8	1,000	0.170	6.2	0	Neutral.
" 8–9	740	0.098	6.6	10	Alkaline.
" 9–10	1,240	0.037	7.1	15	"
" 10–11	805	0.020	5.2	15	"
" 11–12	1,130	0.028	6.3	15	"
" 12–13	1,400	0.160	5.2	0	"
" 13–14	660	0.140	4.3	0	Neutral.
" 14–15	600	0.150	4.2	8	Alkaline.
" 15–16	—	—	—	10	"
" 16–17	800	0.080	5.2	10	"
" 17–18	—	—	—	10	"
" 18–19	600	0.025	4.9	10	"
" 19–20	—	—	—	10	"
" 20–21	860	0.025	5.4	10	"
" 21–22	—	—	—	10	"
" 22–23	980	0.035	5.7	10	"
" 23–24	—	—	—	10	"
" 24–25	920	0.036	5.2	10	"
" 25–26	1,040	0.031	—	10	"
" 26–27	860	0.017	4.1	20	"
" 27–28	1,500	0.025	—	20	"
" 28–Mar. 1	1,380	0.030	—	20	"

diet free from added salt. This ration was varied to suit the individual tastes, but once chosen, the subject was required to adhere to it during, and for 3 days before the experimental period.

For these experiments we have used two cases of nephritis as representing a condition in which the presence of a low grade acidosis is generally held to exist, and in which according to our earlier results the ingestion of alkali leads to the production of a urine of minimum ammonia content; two cases of catarrhal jaundice, a condition in which large doses of alkali produce relatively little effect on ammonia excretion, and which has been designated as a condition in which an abnormal production of acid radicals is present;[1] and two normal persons.

In Experiments VII to XIV we have calculated from the carbon dioxide of the urine the sodium bicarbonate excreted by way of the kidney. These calculated figures are given in the last column under the heading, "Sodium bicarbonate excreted", and probably

EXPERIMENT VI.

Subject 6.—Male. 26 years old. Weight 60 kilos. Acute catarrhal jaundice (convalescent).

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Sodium bicarbonate taken.	Reaction of urine to litmus.
1917	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	
Dec. 21–22	1,180	1.30	0.50		0	Acid.
" 22–23	1,760	1.35	0.48	9.4	0	"
" 23–24	2,530	1.41	0.27	10.4	10	Neutral.
" 24–25	2,460	1.50	0.11	8.2	10	Alkaline.
" 25–26	2,180	1.28	0.21	8.7	10	"
" 26–27	1,100	1.45	0.29	7.8	10	"
" 27–28	2,020	1.31	0.14	7.8	10	"
" 28–29	1,765	1.45	0.10	7.9	15	"
" 29–30	1,960	1.39	0.10	8.2	15	"
" 30–31	1,810	1.75	0.18	8.2	0	Acid.
" 31–Jan. 1	2,040	1.56	0.35	8.6	0	"
1918						
Jan. 1–2	1,840	1.36	0.53	7.9	0	"
" 2–3	2,250	1.37	0.60	10.2	20	"
" 3–4	3,120	1.53	0.29	10.4	20	Alkaline.
" 4–5	2,020	1.41	0.10	7.2	20	"
" 5–6	2,970	1.50	0.08	6.5	20	"
" 6–7	3,210	1.61	0.13	8.5	20	"
" 7–8	3,580	1.47	0.28	8.1	20	"
" 8–9	3,060	1.50	0.17	7.6	20	"
" 9–10	3,260	1.50	0.18	8.5	0	Acid.
" 10–11	2,900	1.50	0.48	7.6	0	"
" 11–12	1,400	1.55	0.36	9.3	0	"
" 12–13	2,500	1.62	0.50	11.0	0	"
" 13–14	2,100	1.47	0.50	10.3	20	Alkaline.
" 14–15	2,200	1.45	0.15	10.3	20	"
" 15–16	1,680	1.42	0.14	9.6	20	"
" 16–17	1,928	1.40	0.16	9.4	20	"
" 17–18	1,680	1.52	0.10	11.1	20	"
" 18–19	1,780	1.38	0.06	12.5	20	"
" 19–20	1,260	1.36	0.23	10.4	0	"

represent maximum values as in the calculation no account has been taken of the alkali carbonate and bicarbonate taken with the vegetable portion of the food.

The figures collected in these tables are instructive as furnishing the explanation of the different behavior of urinary ammonia excretion in the three classes of subjects used. In the first two

cases (Experiments VII and VIII) it will be noted that the rise in carbon dioxide excretion is slow but that the excretion continues at a high level for 2 days after alkaline therapy had been

EXPERIMENT VII.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Jan. 12–13	1,250	1.27	0.112	8.35		9.75	0	
" 13–14	1,380	1.27	0.165	7.59	76	10.60	0	0.28
" 14–15	630	1.21	0.050	7.92	72	8.7	20	0.28
" 15–16	820	1.20	0.049	7.43	1,927	8.5	20	7.2
" 16–17	830	1.23	0.052	7.76	2,378	13.10	20	8.9
" 17–18	740	1.14	0.035	7.42	2,214	12.51	20	8.2
" 18–19	780	1.27	0.010	7.50	2,106	12.06	0	7.8
" 19–20	620	1.21	0.000	7.50	1,336	10.66	0	5.0
" 20–21	400	1.24	0.015	5.90	120	9.9	0	0.45

Mr. B., nephritic, served as subject for Experiment I. The daily ration during the experimental period and for the 3 preceding days consisted of the following: bread 120 gm., shredded wheat 25 gm., potato 150 gm., cabbage 100 gm., eggs 100 gm., milk 750 cc., butter 10 gm., lettuce 15 gm., pineapple (canned) 70 gm., apple 100 gm., orange 150 gm., grape fruit 200 gm., sugar 25 gm.

EXPERIMENT VIII.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Feb. 17–18	800	1.67	0.048	6.40		6.49	0	
" 18–19	1,000	1.53	0.055	6.80	112	6.84	0	0.04
" 19–20	920	1.54	0.025	6.92	184	8.09	15	0.69
" 20–21	800	1.44	0.021	5.32	672	7.84	15	2.51
" 21–22	1,980	1.44	0.021	9.30	3,326	21.84	15	12.43
" 22–23	1,175	1.47	0.014	6.85	1,788	16.69	15	6.69
" 23–24	760	1.37	0.008	6.90	304	11.1	0	1.13
" 24–25	1,100	1.48	0.030	7.20	360	7.3	0	1.34

Mr. M. 48 years old. Weight 72 kilos. Chronic interstitial nephritis. Little edema. Urine contained a slight trace of albumin and a few hyaline casts. The daily ration for the experimental period and for 3 days preceding was similar to that given in Experiment VII.

EXPERIMENT IX.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Jan. 13–14	2,100	1.47	0.500	10.3	157	12.9	0	0.58
" 14–15	2,200	1.45	0.154	10.3	2,212	17.7	20	8.27
" 15–16	1,680	1.42	0.100	9.6	6,048	18.5	20	22.62
" 16–17	1,920	1.40	0.160	9.4	5,376	23.2	20	20.10
" 17–18	1,680	1.52	0.100	11.1	3,360	21.0	20	12.56
" 18–19	1,780	1.38	0.060	12.5	4,350	27.3	20	16.27
" 19–20	1,260	1.36	0.232	10.4	273	10.8	0	1.02
" 20–21	1,400	1.42	0.510		112	12.5	0	0.41
" 21–22	1,300	1.40	*		117	11.2	0	0.44

Mr. F., catarrhal jaundice, served as subject for Experiment VI. The daily ration taken during the experimental period and for 3 days preceding it consisted of the same foods used in Experiment VII with the addition of 3 eggs.

EXPERIMENT X.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Jan. 13–14	1,900	1.09	0.492	12.80	265	11.9	0	0.99
" 14–15	1,700	1.02	0.120	9.10	3,740	12.8	20	13.98
" 15–16	1,660	1.10	0.100	10.54	6,142	10.0	20	22.96
" 16–17	2,000	1.04	0.032	12.20	6,200	23.1	20	23.18
" 17–18	1,900	1.14	0.075	9.11	5,172	27.4	20	19.34
" 18–19	2,140	1.09	2.120	11.23	1,661	27.3	0	6.21
" 19–20	1,920	1.07	0.400	12.12	253	13.6	0	0.94
" 20–21	1,420	1.06	0.500	12.85	157	12.7	0	0.60

Mr. Fn. 43 years old. Weight 49 kilos. Acute catarrhal jaundice (convalescent). The diet was identical with that used in Experiment IX.

EXPERIMENT XI.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Jan. 26–27	1,280	1.23	1.52	21.63	96.0	13.56	0	0.35
" 27–28	1,060	1.26	1.07	17.28	254.4	16.20	10	0.94
" 28–29	960	1.17	0.33	17.37	633.0	17.00	15	2.36
" 29–30	1,080	1.18	0.28	19.98	3,996.0	18.90	15	14.96
" 30–31	1,500	1.27	0.30	21.75	1,800.0	22.87	15	6.73
" 31–Feb. 1	660	1.19	0.90	8.77	520.0	9.72	0	1.94

The subject of this experiment was the same normal person used in Experiment IV. The daily ration for all but the last day of this experiment consisted of the following: bread 200 gm., butter 50 gm., eggs 12, gelatin 50 gm., cheese (Neufchatel) 200 gm., oranges 2, lemons 2. On Jan. 31 severe headache and nausea made it impossible to continue the experiment.

EXPERIMENT XII.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Feb. 7–8	1,080	1.16	0.230	8.10	194	15.49	0	0.72
" 8–9	1,260	1.11	0.150	5.88	1,890	18.90	10	7.10
" 9–10	2,180	1.10	0.170	7.53	3,475	30.08	15	12.99
" 10–11	1,750	1.17	0.087	6.03	3,237	24.06	15	13.11
" 11–12	1,600	1.12	0.035	6.08	3,120	23.20	15	11.66
" 12–13	1,640	1.11	0.186	5.90	543	20.50	0	2.03
" 13–14	1,130	1.15	0.210	5.08	156	15.80	0	0.58

The subject of this experiment was the same normal person used in Experiments IV and XI. The daily ration during the experimental period consisted of the following: bread 150 gm., cornmeal 50 gm., potato 300 gm., bacon fat 75 gm., spinach (canned) 150 gm., apple, orange, and banana each 150 gm., butter 50 gm., cream, 40 per cent, 60 cc.

EXPERIMENT XIII.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*
Jan. 27–28	1,300	1.01	1.23	23.63	26	14.40	0	0.09
" 28–29	1,320	1.13	1.05	23.49	69.2	14.65	10	0.26
" 29–30	1,120	1.11	0.18	20.49	1,568	16.67	15	5.86
" 30–31	1,160	1.16	0.20	19.25	1,624	18.57	15	6.07
" 31–Feb. 1	1,170	0.97	0.14	18.25	1,520	22.66	15	5.68
Feb. 1–2	1,120	1.12	0.45	20.16	448	18.1	0	1.67
" 2–3	1,540	1.05	0.70	21.56	231	19.0	0	0.86

The subject of this experiment was the same normal person used in Experiment V. The daily ration during the experimental period consisted of the following: bread 150 gm., butter 25 gm., milk 500 cc., eggs 12, gelatin 50 gm., cheese (Neufchatel) 200 gm., string beans (canned) 100 gm.

EXPERIMENT XIV.

Date.	24 hr. volume.	Creatinine.	NH_3	Total N.	Total CO_2.	Total ash.	Sodium bicarbonate taken.	Sodium bicarbonate excreted.
1918	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	*cc.*	*gm.*	*gm.*
Feb. 7–8	1,000	1.10	0.170	6.20	180	12.06	0	0.67
" 8–9	740	1.08	0.098	6.66	1,406	16.65	10	5.24
" 9–10	1,240	1.11	0.037	7.13	3,850	24.67	15	14.39
" 10–11	805	1.12	0.020	5.18	3,069	22.16	15	11.47
" 11–12	1,130	1.00	0.028	5.44	3,309	22.30	15	12.37
" 12–13	1,400	1.07	0.160	5.25	1,022	19.70	0	3.82
" 13–14	660	1.10	0.140		159	15.01	0	0.59

The subject of this experiment was the same normal person used in Experiments V and XIII. The daily ration during the experimental period consisted of the following: bread 100 gm., shredded wheat 25 gm., potato 450 gm., bacon 75 gm., spinach (canned) 150 gm., string beans (canned) 100 gm., apple and banana 200 gm. each, butter 25 gm., cream, 40 per cent, 150 cc., olive oil 15 cc.

abandoned, a phenomenon due no doubt to the inability of the kidneys in these two nephritics to get rid of the ingested salts, until continued administration had raised the concentration to a high level. In the prolonged excretion is furnished an explanation of the fact noted in our earlier experiment; *i.e.* that ammonia-free urines will be excreted for several days after the administration of alkali has ceased.

The third and fourth cases (Experiments IX and X) belong to the type of subject in which long continued administration of alkali produces relatively little effect on the ammonia excretion, a finding due apparently to the fact that the alkali is excreted with great rapidity before its full neutralizing effect can be exerted.

In the case of the two normal subjects we have made observations on an alkaline and on an acid diet. These results need little comment as they coincide with what could have been predicted on theoretical grounds. In both cases during the period of high protein feeding less than 50 per cent of the ingested alkali was recovered while in the low protein period the percentage of bicarbonate recovered amounted to nearly 90 per cent of the amount administered.

It would seem that our results may also be applied to explain at least in part the extremely low ammonia coefficients frequently found in nephritis,[7] as it is possible that these low ammonia figures are caused by the relatively slow excretion of the salts constant in the alkaline vegetarian diets, usually furnished to nephritics, and which are therefore able to neutralize more efficiently than in the case of the normal subject the acids arising in the course of the metabolic processes.

SUMMARY.

By the intermittent administration to nephritics of small doses of sodium bicarbonate it has been possible to obtain urines absolutely free from even traces of ammonia. An attempt to duplicate these results on normal subjects has been unsuccessful. Our results furnish support to the theory that the sole use of urinary ammonia is for the neutralization of acids found during the normal metabolic processes, and would seem to render superfluous the speculations regarding the "residual ammonia fraction" of urine.

[7] Palmer, W. W., and Henderson, L. J., *Arch. Int. Med.*, 1915, xvi, 109.

THE CASEIN OF HUMAN MILK.

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The milk used for this investigation was collected each morning by one of us, brought to the laboratory, and treated as follows:

It was placed in separatory funnels, centrifugalized, the fat-free milk withdrawn, and the casein precipitated by the careful addition of 0.6 per cent acetic acid. The precipitated casein was removed from the milk serum, washed several times with water, and purified in the manner previously described by Van Slyke and Bosworth[1] in connection with the preparation of casein from cow's milk except that the treatment with ammonia and ammonium oxalate was omitted because human milk unlike cow's milk does not contain the calcium salts which make this treatment necessary in preparing casein from cow's milk.

The final preparation contained 5.31 per cent moisture and gave the following figures upon analysis. For comparison we also give corresponding figures for the casein of cow's milk and goat's milk.

Nitrogen, Phosphorus, and Sulfur in Caseins.

	Cow[1].	Goat[2].	Human.
	per cent	*per cent*	*per cent*
Nitrogen in dry substance.....	15.80	15.67	15.75
Phosphorus " " "	0.71	0.71	0.70
Sulfur " " "	0.72	0.71	0.70

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 203.

[2] Bosworth and Van Slyke, *J. Biol. Chem.*, 1916, xxiv, 173.

Relation of Casein of Human Milk to Bases.

By applying the details of the methods devised by one[3] of us we find that the casein of human milk combines with bases to form the same series of salts (caseinates) as in the cases of the caseins from cow's milk and goat's milk. Omitting details the results of our work can be briefly summarized as follows:

1. 1 gm. of casein combines with 9×10^{-4} gm. equivalents of base to form a compound (a caseinate) neutral to phenolphthalein.

2. 1 gm. of casein combines with 1.125×10^{-4} gm. equivalents of ammonium, sodium, or potassium to form a soluble acid caseinate, and there is no other combination of casein with base containing less than 1.125×10^{-4} gm. equivalents of base for 1 gm. of casein.

3. 1 gm. of casein combines with 2.25×10^{-4} gm. equivalents of calcium to form an acid caseinate which is soluble in water. This acid caseinate is insoluble in water in the presence of more than a trace of any soluble salt of calcium, barium, or strontium.

4. 1 gm. of casein combines with 1.125×10^{-4} gm. equivalents of calcium to form an acid caseinate insoluble in water. This compound is soluble in a warm 5 per cent solution of sodium chloride, the solvent action being due to an interchange of bases by double decomposition, with the consequent production of soluble sodium caseinate.[4]

Valency and Molecular Weight of Casein of Human Milk.

From the preceding data, it can be seen that the valency of casein from human milk, like that of the caseins from cow's and goat's milks, is 8.

$$\frac{9 \times 10^{-4}}{1.125 \times 10^{-4}} = 8$$

The molecular weight would therefore be $\frac{1}{1.125 \times 10^{-4}} = 8{,}888$ and the equivalent weight would be $\frac{8{,}888}{8} = 1{,}111$.

[3] Bosworth and Van Slyke, *J. Biol. Chem.*, 1913, xiv, 207; Van Slyke and Bosworth, *ibid.*, 211, 227, 231.

[4] Van Slyke and Bosworth, *J. Biol. Chem.*, 1913, xiv, 217.

On the basis of the sulfur content we have:

$$\text{Molecular weight} = N\left(\frac{32.07}{0.70}\right) \times 100 = N\,(4{,}581).$$

If N equals 2, the molecular weight will be 9,162.
On the basis of the phosphorus content we have:

$$\text{Molecular weight} = N\left(\frac{31.04}{0.70}\right) \times 100 = N\,(4{,}434).$$

If N equals 2, the molecular weight equals 8,868.

Action of Rennin on the Casein from Human Milk.

Pure solutions of unhydrolized acid calcium caseinate curdle upon the addition of rennin in exactly the same manner as was found in the case of the casein from cow's milk,[5] the curd consisting of calcium paracaseinates. 1 molecule of casein gives 2 molecules of paracasein. The paracasein thus formed has one-half the molecular weight (4,444) of casein and has a valency of 4, one-half that of casein.

SUMMARY.

A considerable quantity of pure casein has been prepared from human milk. This casein was found to resemble the casein from cow's and goat's milk in the following respects: it has the same nitrogen, phosphorus, and sulfur content; it has the same degree of valency, and gives the same series of salts with bases; it has the same molecular weight; it is acted upon by rennin in the same manner; the paracasein produced by the action of rennin is similar to the paracasein produced by the action of rennin on the casein of cow's milk.

[5] Bosworth, A. W., *J. Biol. Chem.*, 1913, xv, 331; 1914, **xix**, 397.

ON THE PREPARATION OF OVALBUMIN AND ITS REFRACTIVE INDICES IN SOLUTION.

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Numerous workers[1–8] have reported methods for the preparation of egg albumin, but the purest preparations have probably been obtained by Osborne and Campbell by fractionally precipitating with saturated ammonium sulfate solution in the presence of sufficient hydrochloric acid. For the purpose of measuring the refractive index constant of this protein, the writer attempted to follow the description for the preparation of ovalbumin given by Osborne and Campbell, but found that, contrary to their findings, crystals of ovalbumin were not obtained.

Following their fractional precipitation method, to the whites of strictly fresh eggs was added an equal volume of saturated ammonium sulfate solution. The egg white was constantly and thoroughly stirred during the addition of the sulfate solution. If the sulfate solution has been added too rapidly or if stirring has been too feeble, difficulty will be encountered later in precipitating the ovalbumin in the crystalline condition. The globulin precipitate A was filtered off. To the filtrate, saturated ammonium sulfate solution was added until a small permanent pre-

[1] Hofmeister, F., *Z. physiol. Chem.*, 1890, xiv, 165.

[2] Gabriel, S., *Z. physiol. Chem.*, 1891, xv, 456.

[3] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

[4] Osborne, T. B., *J. Am. Chem. Soc.*, 1899, xxi, 477.

[5] Schulz, F. N., *Z. physiol. Chem.*, 1900, xxix, 86.

[6] Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 422.

[7] Cohn, M., *Z. physiol. Chem.*, 1904–05, xliii, 41.

[8] Sörensen, S. P. L., *Chem. Abstr.*, 1918, xii, 913.

cipitate was produced and then for each 6 liters of egg white a mixture of 600 cc. of saturated ammonium sulfate solution, 830 cc. of water, and 27 cc. of concentrated hydrochloric acid were gradually added. According to the method, on allowing the solution to stand over night, a large quantity of fine needle-shaped cyrstals should have separated. Contrary to expectations, no crystals appeared. This led the writer to investigate the cause for the failure to obtain crystals.

It was surmised that failure to secure crystals was due to lack of the proper acidity. This was also indicated by Dr. T. B. Osborne in a private communication. He considered the most critical feature of the whole operation to be the securing of the correct acidity. The writer has found upon investigation that the crystallization of ovalbumin is dependent on an optimum H ion concentration.

To the filtrate obtained after Precipitate A was filtered off, a saturated ammonium sulfate solution was added until a small permanent precipitate was produced. About 25 cc. of this solution were then run into each of several Erlenmeyer flasks. To each of these flasks was then added an arbitrary amount of the acid mixture until in the judgment of the investigator a sufficient amount had been added to cause the formation of crystals. On standing over night, it was found that some flasks contained fine needle-shaped crystals while other flasks contained no crystals.

To these latter flasks a very little more of the acid mixture was added, and the solutions were again allowed to stand over night. In some instances crystals were then formed, whereas in those flasks which showed no crystals it was found that the solution had been made too acid on the first or on the second addition of the acid mixture. The experiments were repeated several times with the hope of obtaining an idea as to the correct acidity necessary for crystal formation. The precipitates were filtered off, and the H ion concentration of the filtrates was determined by means of various indicators. The results show strikingly that the crystal formation of ovalbumin is dependent upon the presence of an optimum H ion concentration.

Fine needle-shaped crystals were obtained when the H ion concentration of the solution in the flasks showed the following reactions:

Indicators.[9]	*Color.*
Alizarin sodium sulfonate	Red.
Rosalic acid	Yellow.
Methyl orange	Yellow.
Cochineal	Lilac.
Congo red	Scarlet-red.
Alizarin	Brown-yellow.
Litmus solution	Violet-blue.
Litmus paper	Bluish red.

No crystals appeared when the H ion concentration of the solutions in the flasks gave the following reactions:

Indicator.	*Color.*
Alizarin sodium sulfonate	Green-yellow.
Methyl orange	Orange-red.
Congo red	Blue.

These results which were repeatedly obtained and which were readily duplicated, indicate that crystals can be obtained at a H ion concentration of from 10^{-5} to 10^{-6}; the lesser acidity gives the best results. When the H ion concentration was 10^{-7} or less (that is when the solution was neutral or alkaline in reaction) it was found that the precipitate was amorphous and contained no crystals. It was found that the optimum amount of the acid mixture could be added to the solution, by simply adding the acid mixture drop by drop while vigorously stirring and noting after the addition of every few drops the reaction of the solution with litmus paper. When the litmus paper showed a perceptible change in color, a sufficient quantity of the acid mixture had been added. It is difficult to prescribe a definite quantity of the acid mixture that must be added to a solution in order to obtain crystals without reference to the H ion concentration, since the alkalinity of egg white appears to vary according to the freshness of the eggs. After the correct acidity has been obtained, fractionation of the ovalbumin can be made by successive additions of saturated ammonium sulfate solution.

The procedure in separating the crystalline ovalbumin and the other fractions was then continued according to the method of Osborne and Campbell. All manipulations, except the

[9] Salm, E., *Z. physik. Chem.*, 1906-07, lvii, 471.

dialysis, were carried out at a temperature of about 10–15°C. without the addition of preservative.

An electric fan, when blowing air of room temperature (about 20°C.) over wide, flat, glass dishes containing the ovalbumin solution, was found to dry the protein rapidly. The process of drying could be hastened by warming this air, but the writer has not found this to be absolutely necessary. When the dialyzed ovalbumin was being evaporated to dryness at a temperature below 50°C. (to prevent coagulation), it was found that as the solution became more concentrated, small particles of seemingly coagulated protein could be seen in the solution. These were centrifuged off.

The amorphous ovalbumin was pulverized in a mortar and thoroughly mixed. The first preparation of ovalbumin was found to have a water content of 8.2 per cent when dried at 110°C. The nitrogen, 15.49 per cent, and ash, 1.24 per cent, content (based on the dry weight of the substance) compares favorably with the results of previous investigators. The second preparation of ovalbumin showed a water content of 8.62 per cent. Its nitrogen content was 15.77 per cent, and ash content 0.14 per cent (based on the dry weight of the substance). The writer has confirmed the observations of Osborne and Campbell that preparations of ovalbumin, obtained by evaporating their clear solutions in pure water to dryness below 50°C., do not completely redissolve when treated with water. This undissolved substance may be a mechanical coagulum or possibly may be due to the effect of any excess of H ions used in the precipitation of the crystals of ovalbumin.

Following similar lines of work in determining the refractive index constants of proteins in solution by various workers[10] in this laboratory and elsewhere, the preparations of ovalbumin were used for the determination of the refractive index[11] constant. Robertson[10] has shown that if proteins are dissolved in an acid or alkaline aqueous solution, the change in the refractive index of the solvent is directly proportional to the concentration of the dissolved protein. This fact can be expressed algebraically

[10] Robertson, T. B., *J. Physic. Chem.*, 1909, xiii, 472; The physical chemistry of the proteins, New York, 1918, 359.

[11] Schmidt, C. L. A., *J. Biol. Chem.*, 1915, xxiii, 487.

as follows: $n - n_1 = a \times c$, where n is the observed refractive index of the solution, n_1 the refractive index of the solvent in which the protein is dissolved, c the percentage of the protein in solution, and a a constant, expressing the change in the refractive index of the solvent by the addition of 1 gm. of protein per 100 cc. Robertson[10] has shown that the refractive index of a solution of sodium caseinate and that of water at the same temperature are independent of the temperature between 20 and 40°C.

The refractive indices of various solutions containing different concentrations of ovalbumin (based on dry weight at 110°C.) were made in a Pulfrich refractometer, reading to within 1′ of the angle of total reflection. A sodium flame was used as the source of light. All solutions were kept for some time in the room so that the temperature of the refractometer prism and that of the solutions used would be the same. Absolute values for the refractive indices are unnecessary in the determination of the constant a, since we are only concerned with the difference

TABLE I.

Preparation 1.

Concentration of ovalbumin.	Refractive index.	a	Possible error for a.
Solvent, water. Temperature 22–23°C.			
per cent			
0	1.33284		
1.0	1.33464	0.00180	±0.00008
1.5	1.33551	0.00178	±0.00005
2.0	1.33639	0.00178	±0.00004
Solvent, 0.1 N NaOH. Temperature 22–23°C.			
0	1.33394		
1.0	1.33575	0.00181	±0.00008
1.5	1.33671	0.00184	±0.00006
2.0	1.33751	0.00179	±0.00004
Solvent, 1 per cent $(NH_4)_2 SO_4$. Temperature 22–23°C.			
0	1.33449		
1.0	1.33623	0.00174	±0.00008
1.5	1.33711	0.00175	±0.00005
2.0	1.33791	0.00171	±0.00004

TABLE II.

Preparation 2.

Concentration of ovalbumin.	Refractive index.	a	Possible error for a.
Solvent, 0.1 N NaOH. Temperature 22–23°C.			
per cent			
0	1.33402		
1.0	1.33583	0.00181	±0.00008
1.5	1.33671	0.00179	±0.00006
2.0	1.33751	0.00175	±0.00004
Solvent, 0.05 N NaOH. Temperature 23°C.			
0	1.33343		
1.0	1.33520	0.00177	±0.00008
1.5	1.33607	0.00176	±0.00005
2.0	1.33695	0.00176	±0.00004
Solvent, 0.0136 N NaOH. Temperature 23°C.			
0	1.33292		
0.909	1.33457	0.00181	±0.00008
1.36	1.33536	0.00179	±0.00005
1.818	1.33607	0.00173	±0.00004
Solvent, 0.005 N NaOH. Temperature 23°C.			
0	1.33292		
0.5	1.33378	0.00172	±0.00016
1.0	1.33472	0.00180	±0.00008

between the refractive index of solution and solvent. In the preceding tables are given the results that were obtained. The values for a are obtained from the equation $n - n_1 = a \times c$.

From the tables it is evident that within the limits of the experimental error and for the different solvents used, the value for a is a constant. The experimental error, reading to within 1′ on the refractometer, is greatest in the most dilute solutions and least in the more concentrated, hence to average the values for a as calculated from each determination would not give the most nearly correct probable value for a. Robertson[12] has shown that the correct method of obtaining the value for a is to add all the values for $n - n_1$ and divide this total by the total

[12] Robertson, *J. Biol. Chem.*, 1910–11, viii, 510.

of the concentrations used. The same method of calculation is likewise employed in determining the possible error for the constant a. Using this method of calculation, we obtain, from all the determinations made, the average value for a as 0.00177 ± 0.00006.

In the case of ovalbumin, as has been observed by previous investigators for other proteins, the increase in the refractive index of the aqueous solvent due to a given concentration of the dissolved protein is independent of the nature and concentration (within the limits studied) of the aqueous solvent.

SUMMARY.

1. The crystallization of ovalbumin was found to be dependent upon the H ion concentration of the globulin-free solution of egg white; crystals are formed when the H ion concentration lies between 10^{-5} and 10^{-6}.

2. The refractive indices of varying amounts of ovalbumin dissolved in water, 1 per cent ammonium sulfate, and in various concentrations of sodium hydroxide, have been measured.

3. The solutions of ovalbumin were found to follow the law $n - n_1 = a \times c$; where n is the observed refractive index of the protein solution, n_1 that of the aqueous solvent, c the percentage of dissolved protein, and a a constant expressing the change in the refractive index of the aqueous solvent by the addition of 1 per cent of protein.

4. The average value for a in the above formula was found to be 0.00177 ± 0.00006; the value obtained by Robertson[13] for the albumins of ox serum being 0.00177 ± 0.00008.

I am indebted to Dr. Carl L. A. Schmidt for the constant interest that he has taken in this work and to the Sheldon Travelling Fellowship of Harvard University which made possible the financial aid necessary in carrying out the investigation.

[13] Robertson, *J. Biol. Chem.*, 1912, xi, 179.

THE PREPARATION OF PURE CASEIN.

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(Received for publication, May 3, 1918.)

The method which has long been in common use for the preparation of casein from cow's milk is the one originally devised by Hammarsten. This consists essentially in separating casein from diluted milk by means of acid, filtering and washing the separated casein, and dissolving it in dilute alkali, after which the processes of coagulation by acid, filtration, washing, and redissolving are repeated four or five times. While modifications in some minor details have been proposed, the preparations of casein obtained by the Hammarsten method and its modifications have been of questionable purity in one or more respects. Such preparations of casein usually contain calcium phosphate, mono-calcium caseinate (the brine-soluble compound), and probably also products of hydrolysis due to exposure of the casein to alkali, acid, and water for unusual periods of time.

In studying the work of Van Slyke and Bosworth[1] it was noticed that soon after milk coagulates in the ordinary process of souring, all of the calcium and inorganic phosphorus of the milk is in solution. This fact suggested the possibility of preparing a pure casein that would not be subjected to the objectionable conditions connected with the Hammarsten process. It occurred to us that if an acid could be introduced into undiluted milk in a manner resembling the formation of acid in the natural souring of milk, the objectionable features could be obviated and a pure product obtained. The most important condition of natural souring to be thus approximated is the gradual addition of acid and its immediate distribution through the mass of milk without causing coagulation of casein at the point where the acid first comes into contact with a portion of the milk. This result can

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1916, xxiv, 191.

be accomplished by introducing the acid below the surface of the milk with simultaneous high-speed mechanical stirring, the tip of the tube that carries the acid into the milk being so arranged that it is very close to the stirrer and near the bottom of the vessel containing the milk (see Fig. 1). The details of the application of this principle have been developed in practical use and are here presented.

I. Method.

1. Description of Apparatus.

The apparatus used in coagulating casein in milk by adding acid under the surface with rapid stirring consists of four main parts: the vessel, the burette, the acid-carrying tube, and the stirrer (see Fig. 1).

Vessel Holding Milk.—The container (A) in which the coagulation of casein takes place may consist of any suitable open vessel, such as a wide-mouthed bottle, a precipitating jar, or an ordinary granite-ironware pail, having a capacity of 1 to several liters according to individual requirements. It is desirable that the vessel should be of relatively low form, since it is more difficult to stir the entire body of the milk satisfactorily if it is in too deep a layer, and also there is a greater tendency to vibration of stirrer.

Burette.—The burette (B) holding the acid is an ordinary one. While only one stop-cock is necessary, two are convenient, one to serve as a shut-off, and the other to regulate the flow of acid.

Tube Carrying Acid.—The capillary glass tube (C), which carries the acid from the burette into the milk, needs detailed description. The diameter of the bore of the tube should be 1.5 to 2 mm. The special construction of the delivery tip (T) of this tube is important. It should be bent upward somewhat and the opening should be flattened and widened in the form of a narrow slit. The object of the slight upward bend is to prevent milk or particles of coagulated casein from being forced into the delivery tube and interfering with the flow of acid by clogging. The upper end of the delivery tube is connected with the burette tip by means of a close-fitting connection of rubber tubing. Care

must be taken not to permit any strain at this rubber joint during the delivery of acid into the milk because such strain may draw milk or particles of casein into the tube, causing stoppage. For this reason a pinch-cock cannot be used in place of a second stop-cock below the burette.

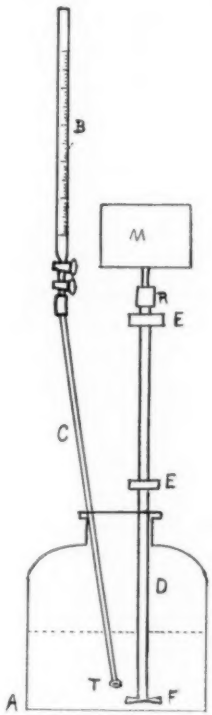

FIG. 1.

Stirring-Rod.—The stirrer (D) is so designed that it will maintain a rapid, uniform speed and stir thoroughly the volume of milk used without producing excessive foam. The stirring-rod is made of perfectly straight, rigid glass tubing about 1 cm. in diameter, and is mounted on two annular ball-bearings (E and E). The attachment of the ball-bearings to the glass stirring-rod is made by interposing sections of rubber tubing of suitable thickness. This arrangement has the effect of keeping the stir-

ring-rod properly centered and thus prevents strain on the glass tubing and consequent vibration and foaming. Connection with the motor (M) is conveniently made by means of rubber tubing (R). Such a stirrer, if properly balanced, is found to run at high speed without vibration and is convenient in the laboratory for many other uses. The motor should be connected with a rheostat to control the speed of the stirrer.

As shown in the illustration, the stirrer is placed in the vessel at one side of the center. In this position the blade (F) forces the milk upward. If the stirrer were in the center of the body of milk, the rotation would tend to form a whirlpool and draw air into the milk, resulting in the formation of foam. Moreover, the upward movement of the milk against the tip of the acid-delivery tube causes the most rapid and thorough mixing of the acid and milk.

The tip of the acid-delivery tube is placed, as shown in the illustration, in such a manner in relation to the revolving blade that the whirling milk washes the end of the tip but is not thrown directly against the opening in the tip in such a way as to force milk or particles of casein into the tube, which might result in clogging the tube and stopping the flow of acid.

2. Milk Used.

Fresh, undiluted milk, the fat of which has been removed by centrifuge, cooled to 15°C., is used. While any amount of milk can be taken by furnishing the required conditions for proper manipulation, the description here given applies, for convenience, to the use of 1 liter of milk.

3. Kind of Acid.

While many acids can be used, our work has been limited to the use of acetic, lactic, and hydrochloric acids. Preference, however, as the result of experience in this work, is given either to normal lactic acid or to a mixture consisting of 1 part of normal hydrochloric acid and 1 or 2 parts of normal acetic acid. It has been found that acetic alone does not always give a satisfactory coagulation of casein in undiluted milk. Satisfactory preparations have been made by the use of hydrochloric acid alone. The only objection to its use is the danger of adding too much acid.

4. Addition of Acid and Coagulation of Casein.

To 1 liter of milk, normal acid is added in the apparatus described above, the stirrer rotating as rapidly as possible without causing excessive foaming, which is usually at the rate of 2,000 to 3,000 revolutions per minute. The acid is allowed to flow into the milk at a rate which will not cause appreciable coagulation as shown by application of the following sedimentation test.

A few cc. of the milk are whirled in a sedimentation tube for a short time. If, on pouring the liquid from the tube, a sediment remains at the bottom, it is an indication that acid has been added too rapidly and has caused some coagulation of casein at the point of contact of acid and milk. Such action can also be avoided by having the milk and acid at low temperatures. Generally speaking, the first 45 cc. can be added in 30 minutes. The addition of acid is continued until the amount reaches about 60 cc., after which the rate is decreased somewhat until the coagulation point is nearly reached, as shown by the test given below; and then, before addition of more acid, the mixture is allowed to stand about 3 hours, the rate of stirring being diminished to 500 revolutions per minute or less.

The approach to the point of coagulation of casein can be easily ascertained by diluting a few cc. of the acidified milk with an equal volume of distilled water and centrifuging in a sedimentation test-tube, when the casein completely separates. This is due to the fact that the hydrogen ion concentration of acidified milk is increased by dilution and that this increase is sufficient, in case of milk near the point of casein coagulation, to throw down the casein. When this test results in casein separation, the addition of acid is stopped, as stated above. When this test shows no casein separation, then addition of acid is continued and the test repeated at intervals until such separation of casein occurs.

During the latter stages of addition of acid, foaming may make some trouble. This, however, can be obviated or controlled by adding a few drops of octyl alcohol, or by using a stirrer free from vibration, or by having the vessel that contains the milk about double the capacity required to hold the amount of milk used, which will usually provide sufficient room to accommodate the foam; any foam forming subsides promptly when the coagulation of the casein takes place.

After standing under gentle stirring for 3 hours with acidity just below the point of casein coagulation, addition of acid is continued slowly, accompanied as before by rapid stirring in order to obtain the particles of casein coagulum in the finest possible state of division. Addition of acid is now continued to the point at which a portion of the mixture, when centrifuged, gives a definite supernatant layer of solution above the casein. Ordinarily, it requires about 90 cc. of normal lactic acid or a mixture of 1 part of normal hydrochloric acid and 2 parts of normal acetic acid to accomplish this for each liter of milk used. Less normal hydrochloric acid (about 75 cc.) is required. When the coagulation is complete, as shown by the test, the mixture is allowed to stand 2 to 4 hours with gentle stirring.

5. Treatment of Casein Coagulum.

The mixture of coagulated casein and solution is placed in two or four half-liter centrifugal bottles and whirled in a centrifuge at a rate of about 1,000 revolutions per minute until the casein settles at the bottom, occupying about one-third of the original volume of the mixture. The supernatant liquid is decanted. The casein, which is now a white, dense, gelatinous-appearing mass is next washed with distilled water in the centrifugal bottle. In order to mix the water thoroughly with the compacted mass of casein, it is necessary to add the water in small amounts and beat the mixture with a rubber-tipped, glass-rod stirrer into a mass of smooth, creamy consistency, free from lumps; enough water is used to bring the mixture to the capacity of the bottle. This mixture of water and casein is centrifuged until the casein is separated as before, and then the supernatant liquid is decanted. The operation of washing with distilled water is repeated until the casein fails to separate completely from the water when the mixture is centrifuged, indicating freedom from electrolytes. This may require four or five washings. After final washing with water and decantation, the casein is washed first with cold 95 per cent alcohol twice, and then with ether three times, in the same manner as with water, care being taken to add the liquid gradually with vigorous beating, particularly in the first washing with alcohol, in order to make a smooth, homogeneous mix-

ture and prevent aggregations of casein particles. The centrifuging with alcohol and ether does not usually require as long a time as with water. The object of washing with alcohol is to remove the water and any alcohol-soluble material as completely as possible before treating with ether. An excessive amount of water in the casein causes it to cake hard in drying. Washing with ether serves chiefly to remove any fat that remains. A very minute amount of fat in the casein causes a cloudy or milky appearance when the casein is dissolved in excess of lime-water. The casein is finally spread on a smooth, flat surface in the open air to dry. While drying, the mass should be gently worked with a spatula in order to keep it in its finely pulverulent condition.

II. Control of Ash and Phosphorus Content of Casein.

The ash and phosphorus content of casein prepared in the manner described depends upon certain details in the method of preparation. Holding the casein in the uncoagulated condition at a degree of acidity just below the point of coagulation results in a preparation low in phosphorus, the minimum amount being 0.80 per cent. This result is due to the fact that the suspended inorganic phosphate ($CaHPO_4$) in milk is completely dissolved by the treatment. It requires in our experience 3 hours at least to make sure of complete solution of the insoluble phosphate. It is, however, not advisable to let the casein stand in this uncoagulated condition more than 6 hours, especially if not kept at a temperature of 18°C. or lower, because some slight hydrolysis of the casein may occur, which tends to affect unfavorably the properties of the preparation.

If casein after coagulation is held in suspension at a hydrogen ion concentration above the isoelectric point, the amount of ash is low, ranging from 0.05 to 0.15 per cent. This result is due to the decomposition of the acid calcium caseinate (mono-calcium caseinate, soluble in warm, dilute brine solution), the acid combining with the calcium and leaving the casein base-free. Complete action requires 2 to 4 hours. Longer standing under these conditions is not likely to cause any hydrolysis, according to our experience. When, therefore, it is desirable for convenience to carry the preparation over night and complete it the 2nd day,

the casein can remain in this completely coagulated condition without affecting its character.

When the insoluble inorganic phosphate is not completely dissolved before coagulation, it does not appear possible to remove it completely after coagulation, no matter how long the mixture is allowed to stand. This is indicated by the results embodied in the following table:

Time of standing before coagulation.	Time of standing after coagulation.	Ash.	Phosphorus.*
hrs.	*hrs.*	*per cent*	*per cent*
0	6	0.15	0.85
0	24	0.14	0.86
0	24	0.15	0.83
3	0	0.46	0.81
4	0	0.43	0.81
1	12	0.15	0.85
3	15	0.05	0.80
3	18	0.11	0.81
4	4	0.10	0.81
16	4	0.10	0.81
30	10	0.10	0.80

* We are indebted to Mr. R. W. Keeler for repeating and confirming these determinations.

The first three series of results in the table show that when the mixture is not allowed to stand awhile in the uncoagulated condition at a point of acidity just short of coagulation, the phosphorus content is relatively high. The cases in which the mixture was allowed to stand 3 and 4 hours before coagulation but not at all after show high ash but low phosphorus content, indicating removal of inorganic phosphorus and retention of calcium in the form of mono-calcium caseinate. The last five series of results in the table, from preparations obtained by allowing the mixtures to stand 3 to 30 hours before coagulation and 4 to 18 hours after, show a minimum of both ash and phosphorus. In no case was it found possible to obtain a preparation with a percentage of phosphorus lower than 0.80 by prolongation of the period of standing.

III. Advantages of Method of Making Preparation of Casein.

In comparing the method described in this article with other methods employed for the preparation of casein, there are certain facts in favor of the new method, to which attention is called.

1. By the new method a preparation of casein can be made within 10 hours. Other methods usually require 2 or 3 days.

2. This method uses the milk without dilution and saves much time and labor in lessening the amount of material to be handled.

3. In this method unnecessary excess of acid is avoided. The hydrogen ion concentration of the coagulation mixture is only slightly above the isoelectric point of casein; that is, about pH 4.5 to 4.6.

4. The contact with reagents need not exceed 6 to 8 hours.

5. At no time is the casein brought into contact with alkali.

6. The casein thus prepared is base-free, containing no calcium phosphate and no form of calcium caseinate.

7. The dried casein we obtain forms a very fine powder even without grinding; usually about 90 per cent will pass a screen containing 100 meshes to the inch.

8. This casein very easily and quickly dissolves to a water-clear solution in dilute mono-basic alkali solutions and also in excess of lime-water. Lime-water solutions of the casein, when neutral, are opalescent.

9. By this method it is possible to obtain without appreciable loss all the casein in the milk in pure form. In other methods the losses of casein in manipulation are considerable and unavoidable.

SUMMARY.

1. Casein in pure form, free from inorganic phosphorus, calcium, and hydrolytic products, is prepared by treating undiluted milk with normal acid, preferably lactic or a mixture of 1 part of hydrochloric and 2 parts of acetic. The acid is introduced slowly into the undiluted milk below the surface, the tip of the tube carrying the acid into the milk being so arranged that it is very close to a mechanical stirrer revolving at a high speed and also

near the bottom of the vessel containing the milk. Under these conditions the acid does not cause coagulation of the casein at the point where the acid first comes into contact with a portion of the milk. Details of precipitation, washing, and drying of the casein are given on pages 131 and 132.

2. Ash and phosphorus contents are unusually low, being about 0.10 per cent of ash and 0.80 per cent of phosphorus, with no calcium.

3. By this method casein can be prepared within 10 hours. Excess of acid with danger of hydrolysis is avoided. Contact with reagents is reduced to a minimum. The product is a very fine white powder. It contains neither inorganic phosphorus nor calcium. It dissolves at once in dilute solutions of monobasic alkalies and also in excess of lime-water to a clear solution. Lime-water solutions of this casein, when neutral, are opalescent. The yield of the casein from milk is practically quantitative.

A METHOD FOR MAKING ELECTROMETRIC TITRATIONS OF SOLUTIONS CONTAINING PROTEIN.

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While carrying on an investigation relating to the amount of free lactic acid present in milk during the process of souring,[1] we found it desirable to make many electrometric titrations, giving accurate results, with solutions containing milk proteins, for the purpose of obtaining numerous data to be used in preparing a curve for each series of titrations. The methods previously used for this purpose[2–8] in connection with proteins have involved the preparation of a separate solution and the replating of the electrode for each point in a curve. Such a procedure requires large expenditure of time, labor, and material and, moreover, gives inaccurate results when the proteins coagulate.

We have undertaken to devise a method by which electrometric titrations can be accurately performed in one solution. Hildebrand[9] has proposed a method which partially accomplishes this purpose but the results obtained are of only approximate accuracy, and, moreover, the method is not applicable to solutions containing protein. McClendon[10] has recently published a method of

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 147.

[2] Bugarszky, S., and Liebermann, L., *Arch. ges. Physiol.*, 1898, lxxii, 51.

[3] Robertson, T. B., *J. Physic. Chem.*, 1910, xiv, 528.

[4] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

[5] Pauli, W., and Hirschfeld, M., *Biochem. Z.*, 1914, lxii, 245.

[6] Clark, W. M., *J. Med. Research*, 1914–15, xxxi, 431; *J. Infect. Dis.*, 1915, xvii, 109.

[7] Schmidt, C. L. A., *J. Biol. Chem.*, 1916, xxv, 63.

[8] Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 371.

[9] Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 847.

[10] McClendon, J. F., *J. Biol. Chem.*, 1918, xxxiii, 19.

continuous electrometric titration, which, however, cannot be used in the presence of proteins that are precipitated by the titration reagent or on the electrode, especially at the point of contact at the surface of the liquid.

I. Apparatus.

The apparatus used by us in making numerous electrometric titrations in one solution consists of several essential parts, as shown in Fig. 1. The new features devised by us relate to the assemblage of the different parts in the vessel in which the reaction takes place and to the arrangement of the electrode. The other parts of the apparatus are the tube carrying the standard acid into the solution, the tube for introducing special reagents, the stirrer, and the tube carrying the saturated KCl solution.

1. Vessel Holding Protein Solution.—The vessel (V), in which the electrode is placed and in which the reaction occurs, is a wide-mouthed bottle. It may be of any size adapted to one's convenience but should have about twice the capacity of the liquid to be used. In our work we have found it convenient to take 100 cc. or 200 cc. of solution, and we therefore use a bottle holding about 400 cc., calibrated in units of 50 cc. The apparatus is so arranged that it can be used in a water-bath if desired.

The neck of the bottle is provided with an easy-fitting cork stopper (S), through which pass into the bottle the several parts or connections of the apparatus. For greatest convenience this stopper is divided into two equal parts, as shown in the illustration, with perforations as indicated. This arrangement simplifies the assemblage of the parts of the apparatus.

2. Hydrogen Electrode.—The hydrogen electrode (A) is suspended inside the *hydrogen-carrying tube* (E). The latter is made from a 10 cc. pipette, which is cut in two in the middle, and the lower part of one side is cut off diagonally, thus forming a bell-shaped projection at the lower end, which is used for the protection of the hydrogen electrode when the parts of the apparatus are being put together. The upper end of this hydrogen tube passes through a hole (E) in the stopper, being fitted so that it can be raised and lowered, thus permitting the lower end to be

raised above, or submerged below, the surface (L–L) of the solution in the vessel, according as it is desired or not to allow hydrogen to bubble through the solution. The tube is connected at the upper end with the hydrogen supply by means of pure gum rubber tubing, which must make gas-tight joint connections.

The hydrogen electrode (A) consists of a piece of platinum foil about 1 cm. square, welded to a platinum wire about 15 cm. long

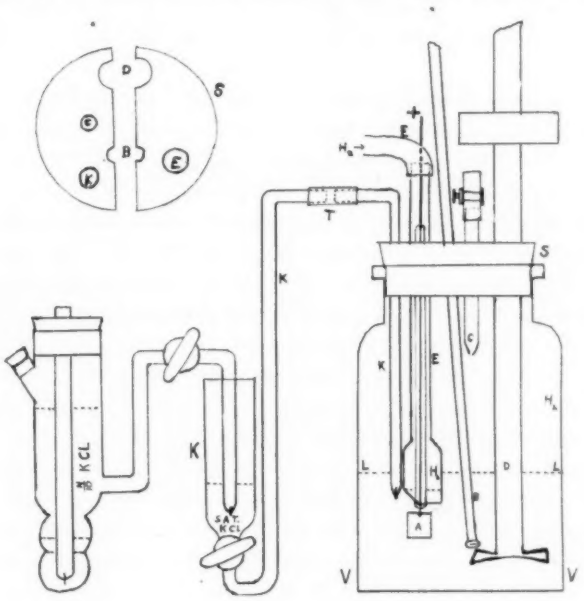

FIG. 1.

The lower half of the wire is protected by enclosure in a piece of glass tubing about 8 cm. long, reaching close to the electrode, and is sealed by fusion at each end. This protecting glass tube must be small enough to pass freely inside the hydrogen tube (E) in order to permit raising and lowering and also to allow the free passage of hydrogen.

In order to arrange the hydrogen electrode properly inside the hydrogen tube, the rubber tubing at the upper end of the hydrogen tube is pulled across the end of the tube and punctured with

a pin; through the hole thus made the upper end of the wire on the platinum electrode is passed making a gas-tight sliding joint. The wire end of the electrode is introduced into the lower or bell-shaped end of the hydrogen tube and is then passed through the pin-hole made in the rubber tubing. The electrode can now be raised or lowered within the hydrogen tube by taking hold of the portion of the wire extending above the rubber tubing. When the electrode is raised so as to be within the bell-shaped projection of the hydrogen tube, it is completely protected from the outside, but it must not touch the inside walls of the bell at any point.

Before using, the electrode is cleaned in hot chromic acid, washed with water, and given a uniform coating of platinum black.[11] It is then dipped again in hot chromic acid, washed, and electrolyzed according to Clark's directions. The bubbles should come from the electrode at all points with perfect uniformity. When this test is satisfactorily met, the electrode is drawn up into the bell and placed in a beaker of distilled water to soak for not less than half an hour, after which it is ready for use. Electrodes thus prepared rarely fail to give satisfactory results. If it is desired to check specially the accuracy of an electrode before using, this is done by any of the standard solutions such as Sörensen's phosphate mixture[12] or Clark's standards.[13]

3. Glass Tube Carrying Titration Reagent.—The capillary glass tube (B), which carries the titration reagent from a burette into the solution in the vessel, has been previously described by us.[14] A burette 30 cm. long and holding 10 cc. can be used to advantage. The hole in the cork through which B passes should be sufficiently close-fitting to hold it firmly.

4. Stirring-Apparatus.—The stirring-apparatus (D) has been already described by us in another paper.[14] The stirring-rod should not fit too closely in the opening in the cork stopper through which it passes but should be allowed room enough for free movement.

[11] Clark, *J. Biol. Chem.*, 1915, xxiii, 475.
[12] Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
[13] Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.
[14] Van Slyke and Baker, *J. Biol. Chem.*, 1918, xxxv, 127.

5. Tube for Introducing Special Reagents.—The special tube (C) is ordinary glass tubing about 2 inches long, which can be used for the introduction of any special reagent that may be desired, such, for example, as caprylic alcohol, when necessary, to prevent foaming. To the upper end of this tube is fitted a piece of rubber tubing, provided with a clamp with which to keep the tube closed when not in actual use for admitting a reagent. By making this tube long enough to reach below the surface of the solution, it can be used for withdrawing solution when desired. In this case the tube should be located near the side wall of the bottle away from the stirrer.

6. Tube Containing KCl.—The tube (K) containing saturated KCl solution consists of a small cylindrical funnel-tube fitted with a glass stop-cock and bent in the manner shown in the illustration. For convenience in handling, the tube is divided at the top (T), and the two separate parts are connected closely with rubber tubing. In case the apparatus is used with a water-bath, the stop-cock should be located near the rubber connection. The end of the tube where it dips into the protein-containing solution in the bottle, is drawn out to make the opening small and this opening is plugged with a small roll of filter-paper to retard the flow and diffusion of the KCl solution. If desired, the saturated solution of KCl can be held in the tube with agar. The level of the KCl solution in the cylindrical funnel must be slightly below that of the liquid (L–L) in the bottle in order to prevent flow. A balance of the solutions is desirable. This tube is fitted in the cork stopper so that it can be raised in order to remove the lower end from the solution. The stop-cock should be closed when the tube is being raised.

7. Additional Pieces of Apparatus.—In making electrometric titrations, the following additional pieces of apparatus are used. (*a*) A Leeds and Northrup potentiometer, type K; (*b*) a Leeds and Northrup galvanometer, type R, for zero instrument; (*c*) a one cell storage battery to supply the working current, which is checked with a Weston standard cell kept at constant temperature. The current being measured originates in the chain, Hg | HgCl | 0.1 N KCl | saturated KCl | solution | H_2 | Pt |, kept at constant temperature during each titration.

II. Advantages of Apparatus.

In addition to the points of convenience already mentioned, the special arrangement of the apparatus in the vessel (V), described above, enables one to overcome certain difficulties that are commonly encountered in making electrometric titrations, such as interference by dissolved oxygen, local chemical action at the point where the titration reagent is introduced into the solution titrated, foaming of solution, deposition of protein on electrode, and interference by bacterial action or by autohydrolysis during titration. These difficulties have been satisfactorily overcome.

1. Interference by Dissolved Oxygen.—This is prevented by displacing the air from the vessel (V) and maintaining a slow current of hydrogen through it.

2. Local Chemical Action.—Chemical action at the point where the reagent is introduced into the solution is prevented by using the specially designed tip and stirrer described by us in another article.[14] The use of the stirrer has been found to be of service also in overcoming some of the other difficulties.

3. Foaming of Solution.—This is a troublesome feature connected with the titration of solutions containing protein. The mechanical stirrer tends to increase the difficulty. It is overcome by introducing the hydrogen above the surface of the solution to be titrated and also by controlling the vibration and speed of the stirrer. While the foaming is thus kept at a minimum, yet enough hydrogen is stirred into the solution to keep the electrode saturated. In case foaming is not troublesome, the hydrogen can be introduced under the surface of the solution in the manner indicated in the illustration. With solutions that foam easily, caprylic alcohol can be used effectively[15] and without interfering with the accuracy of the results of titration. However, its use is advocated only as a last resort.

4. Deposition of Protein on Electrode.—This can be prevented by complete immersion of the electrode in the solution. The rapid movement of the solution prevents local chemical action at the electrode and at the same time hydrogen is stirred into the solution and carried to the electrode. However, other precautions to prevent deposition of protein are essential. The

[15] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 282.

platinum wire attached to the electrode must be protected at the point where it enters the solution, which is accomplished by sealing it into the glass tube, as already described. The electrode after being plated must be soaked in distilled water for a half hour or more before being used, in order to remove adsorbed acid.[3] The apparatus is so arranged that the electrode can be quickly lowered into the solution after the parts of the apparatus are assembled and the stirrer started to rotating. This arrangement prevents collection of protein on the electrode.

5. Bacterial Action and Autohydrolysis.—Interference from these actions can be satisfactorily prevented only by fairly rapid work. We have been able within 2 hours to complete determinations yielding values for use in making a curve, and during such a brief period of time no appreciable interference takes place due to changes caused by these agencies. We have found no antiseptic wholly free from objection when used in the solution under examination. Of those tried, thymol has been found the least objectionable.

III. Operation.

The solution to be titrated is poured into the bottle or vessel (V) and water is added to make the desired volume. If a thermostat is used, the temperature of the solution should now be adjusted. Any bubbles present should be removed, which can be done by pricking them with a greased pin or by touching them with a fine capillary tube containing ether. The burette must be previously filled and all bubbles carried out of the delivery tube (B), the tip of which should be rinsed before it is put into the vessel. The filled delivery tube and the stirrer are placed in position within the vessel or bottle. The two halves of the cork stopper are now placed in position together with the other parts. Care is taken to have the electrode drawn up within the protecting bell so that it does not touch the apparatus or solution. Hydrogen is now permitted to flow rapidly in until the air is displaced, after which the stirrer is set in motion. This precaution is necessary because any bubbles of air that are stirred into the solution greatly retard the attainment of equilibrium. The electrode is now quickly lowered until it is entirely under the

surface of the solution, and connections are then completed for the electrolytic circuit. Equilibrium is quickly reached ordinarily, usually in 2 to 5 minutes after introducing the electrode. During the period approaching equilibrium, the stirrer should be run fast enough to keep a few bubbles of hydrogen constantly in suspension in the solution. Equilibrium is indicated by a constant E. M. F. for 2 minutes or more. When the E. M. F. is satisfactory, the desired amount of reagent is slowly introduced from the burette, during which the stirrer may be slightly accelerated to prevent coagulation but not fast enough to produce foam. After introduction of the reagent, readings are made once a minute until constant. When the amount of reagent introduced is 0.5 cc. or less, equilibrium should be immediate. Titration is now continued until the desired number of values is obtained.

In order to avoid marked dilution of the protein solution, titrations are made with use of N solutions of reagents, and thus the need of making corrections is avoided since the hydrogen ion concentration of the buffered solutions is inappreciably changed by the small degree of dilution under such conditions. The speed of the stirrer must be carefully regulated so as to cause little or no foam; consequently, the addition of the reagent must be moderately slow; for example, about 1 cc. in 2 minutes in the case of N HCl with solutions containing 1 per cent of casein.

The accuracy of the electrometric titration can be checked, when completed, by redetermining the final E. M. F. value of the titration of the solution with a Clark electrode. If agreement is not close, the results of the titration should be discarded and the operation repeated. In our work agreement is nearly always obtained.

SUMMARY.

A method has been devised by which numerous electrometric titrations can be accurately performed in one solution in the presence of proteins.

The apparatus consists of a wide-mouthed bottle, holding about 400 cc., in which the electrode is placed and in which the reaction occurs. The neck is provided with a cork stopper containing perforations through which pass into the bottle the several parts or connections. The hydrogen electrode, made of platinum

foil, is suspended in a special manner inside the bell-shaped end of the tube carrying hydrogen and is so arranged that it can be lowered into, or drawn up out of, the solution titrated. Other parts are (*a*) a glass tube carrying the titration reagent from a burette into the solution titrated, (*b*) a special stirring-apparatus, (*c*) a tube for introducing additional special reagents, (*d*) a tube containing KCl and, in addition, other pieces of standard apparatus required to measure hydrogen ion concentration.

The apparatus has the advantage of enabling one to prevent (*a*) interference by dissolved oxygen, (*b*) local chemical action at point where the reagent enters the solution under titration, (*c*) foaming of solution, (*d*) deposition of protein on electrode, and (*e*) interference by bacterial action or hydrolysis.

In operating, the solution under examination is placed in the bottle, the volume and temperature are adjusted, and all bubbles are removed. The parts of the apparatus are assembled in position, the electrode being above the solution. Hydrogen is run in freely and air displaced. The stirrer is set in motion. The electrode is lowered into the solution, electric connections are made, equilibrium is established, and then N titration reagent run in. Readings of E. M. F. are made once a minute until constant. Titrations are made until the desired number of values is obtained. The method permits rapid work.

FREE LACTIC ACID IN SOUR MILK.

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It has been the universal custom to speak of the acid constituents present in sour milk as lactic acid, just as if the acidity were due directly and entirely to the presence of free lactic acid, as such. The usual method of determination has been to titrate the milk to phenolphthalein with 0.1 N alkali and to express the results either as percentage of lactic acid or as the number of cc. of 0.1 N alkali required to neutralize 100 cc. of milk.

When milk sours under ordinary conditions, lactic acid is first formed but this reacts at once, practically as fast as formed, with the basic constituents present in the milk.[1] In fresh cow's milk, the compounds reacting with acids are, first, basic phosphates and citrates of calcium, magnesium, sodium, and potassium, and second, calcium caseinate.[2] When the lactic acid that is formed in the souring of milk reacts with these compounds, there are formed mono-basic or acid phosphate and citrate salts, free casein, and lactate salts, especially of calcium. It is sufficient for our purpose to represent more simply the essential results of the reactions as consisting of the formation of mono-calcium phosphate, free casein, and calcium lactate. In view of this condition, it cannot be expected that there will be considerable amounts of free lactic acid in souring milk until these reactions are practically completed.

In previous work carried on in this laboratory, efforts were made to determine separately the amounts of free lactic acid and combined lactic acid. It was found, however, in a solution containing lactic acid, that lactates and mono-basic phosphates

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1916, xxiv, 191.

[2] Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 135.

undergo mutual reaction, forming free lactic acid and di-calcium phosphate. It was impossible to tell with accuracy at what point the conversion of lactate into free lactic acid begins, and no satisfactory method by continuous extraction has been found for making a separate determination of free lactic acid in the presence of lactate and phosphate salts.

In the work described in this article, three methods have been used for determining the amount of free lactic acid in sour milk. In two of these methods, the results are based largely upon the measurement of hydrogen ion concentration (C_H) and in the other upon partial extraction of lactic acid by ether. In addition, it has been necessary to make a study of the influence exerted by adsorption of lactic acid by casein. Further work is presented in relation to the effect of different conditions upon the amount of free lactic acid in sour milk, the coagulation point of casein in sour milk, and the first indications of sour flavor in milk.

Methods for the Determination of Free Lactic Acid in Sour Milk.

I. Measurement of C_H and Application of Mass Law.

The free lactic acid that is formed in the souring of milk consists, in part, of ionized and, in part, of un-ionized acid. The ionized, or chemically active portion, combines promptly with the basic constituents of the milk to form lactate, as already pointed out. In accordance with the law of mass action, when a portion of ionized acid is thus removed, a portion of the un-ionized or non-dissociated acid changes into the ionized form, and this change goes on continuously during the process of souring. The amount of ionized lactic acid present at any stage of the souring process is very small in comparison with the un-ionized portion, as shown in Table I.

The lactic acid in sour milk is present, therefore, as free lactic acid and combined lactic acid or lactate, the free lactic acid being present as ionized and un-ionized acid. In accordance with the law of mass action, there exists, under the conditions present in sour milk, definite quantitative relations between the amounts of free lactic acid, ionized lactic acid, and combined lactic acid. Our problem is to make use of these mathematical relations in ascertaining the amount of free lactic acid in sour milk at any

stage of the souring process. These relations can be expressed by the following equation: $\frac{a(b-x)}{x} = k_t$, in which a equals the hydrogen ion concentration (C_H) or ionized lactic acid; b, the total amount of lactic acid formed; x, the amount of un-ionized free lactic acid; and k_t, a constant varying with temperature. In this equation, a is ascertained directly by the measurement of the C_H value; b is found by titration; and k_t ($t = 25°C.$) is about 25 or 26×10^{-5}, as determined by us. Therefore, all the values are known except that of x, the amount of free lactic acid, the value of which is the one we wish to determine. The equation being rearranged becomes $x = \frac{ab}{a + (25 \times 10^{-5})}$.

The value of a (C_H) is determined by means of the hydrogen electrode at 25°C., using Cullen's modification of the Clark cell[3, 4] of 2 cc. capacity, with 0.1 N KCl, calomel half-cell, and saturated KCl for contact. A Weston cell, calibrated by the U. S. Bureau of Standards, is used in conjunction with a 2 volt storage battery as standard voltage. All of the apparatus is kept in an air-bath at 25°C. The hydrogen used is purchased in stored cylinders and purified by passing through strong solutions of alkaline $KMnO_4$ (twice), and alkaline pyrogallol (twice), then dilute H_2SO_4, and finally through a long tube filled with absorbent cotton. The voltage is read with a Leeds and Northrup potentiometer, with a sensitive galvanometer for zero instrument.

The value of b, the total amount of lactic acid formed at any stage in the souring process, is determined by titration to phenolphthalein with 0.1 N NaOH; the figure thus obtained is corrected by the subtraction of the titration value given by the fresh milk.

The value of k at 25°C. is determined with the hydrogen electrode, using a solution of pure lactic acid of known concentration and also solutions of lactic acid containing different amounts of calcium lactate. k is found to have the same value with or without calcium lactate. This method is used in preference to conductivity methods because it is desired to use the constant in mak-

[3] Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 480.
[4] Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 371.

ing interpretation of the results obtained by the same method, expressed in terms of hydrogen ion concentration (C_H). The value of k at 25°C. found by us within the limits of acidity present in sour milk is 25×10^{-5} to 26×10^{-5}. We have not been able to confirm the value for k (14×10^{-5}) found by Ostwald[5] nor that ($k = 31 \times 10^{-5}$) published by Goldschmidt and Bürkle.[6] Van Slyke and Van Slyke[7] found the value considerably higher than that of Ostwald.

EXPERIMENTAL.

It now remains to give some additional details as to how the work was performed in measuring the hydrogen ion concentration (C_H) value and the total amount of lactic acid formed in sour milk.

Fresh milk is run through a centrifugal separator to remove fat and is pasteurized by holding ½ hour at 62°C. A portion is inoculated with a pure, uncurdled culture of *Bacterium lactis acidi* (1:10,000).[8] The inoculated milk is kept at 25°C. By actual testing it was found that the culture remained pure throughout the duration of each experiment. Before inoculation and at intervals after, portions are removed with a sterile pipette and the C_H values immediately determined; this requires about 15 minutes. The results are stated in values of pH and C_H. The temperature is kept at 25°C. during the whole operation.

In each case, the titration with alkali to determine the total amount of lactic acid that has been formed is made immediately before the E. M. F. reading. Thus both determinations are practically simultaneous and any possible error due to difference in time is inappreciable. The titration is made as follows: 0.1 N NaOH is added to 10 cc. of milk and 1 cc. of alcoholic solution of phenolphthalein until the first pink color appears and remains about 60 seconds. The milk is measured with a rapid-delivery pipette calibrated to contain 10 cc. The milk adhering to the

[5] Ostwald, W., *Z. physik. Chem.*, 1889, iii, 193.

[6] Goldschmidt, H., and Bürkle, E., *Ber. chem. Ges.*, 1899, xxxii, 364.

[7] Van Slyke, L. L., and Van Slyke, D. D., *Am. Chem. J.*, 1907, xxxviii, 400.

[8] This and the other cultures referred to later were furnished by Mr. G. L. A. Ruehle, Assistant Bacteriologist at this Station.

inner wall of the pipette is rinsed out with 10 cc. of distilled water, the rinsings being added to the sample to be titrated. With curdled milk the same procedure is followed, except that the 1 cc. of alcoholic solution of phenolphthalein is not added to the milk until sufficient 0.1 N alkali has been added to dissolve most of the curd. This modification is made for the purpose of preventing the hardening effect of the alcohol upon the particles of casein, since such hardening makes it difficult to reach a sharp end-point reaction. With this modification, duplicate determinations in case of curdled milk can be obtained with an agreement within 0.05 cc.

The results of the work are embodied in Table I. In Column 1 the figures indicate the intervals of time between inoculation and sampling for determination. The results in Column 2 are obtained by direct titration in the manner already described, while the figures in Column 3 are found by subtracting the titration result of the fresh milk (18 cc.) from that found in the sample taken at each indicated interval of time. The values in Column 4 are obtained by direct determination, as already explained. The figures in Column 5 are the C_H equivalents of the pH values given in the preceding column and are used in calculating the results given in Column 6. The figures in Column 6 are obtained by use of the equation $x = \frac{ab}{a + k_l}$ in which all values are to be expressed in mols (the molecular weight in gm.) and k_l is taken as 25×10^{-5}. The values for a are based upon the figures in Column 3, while the values of b are given directly in Column 5. The figures in Column 7 are placed here as a matter of convenience for future reference.

The data embodied in Table I can be summarized in the following statements:

1. The total acid formed in the milk in the process of souring, as determined by titration and expressed as cc. of 0.1 N alkali required to neutralize to phenolphthalein 100 cc. of milk, varies from 1 cc. at 15 hours after inoculation to 97.3 cc. 48 hours after inoculation.

2. The pH value of souring milk at different intervals runs from 6.50 (equivalent to C_H, 0.032×10^{-5}) in fresh pasteurized milk to 4.17 (equivalent to C_H, 6.8×10^{-5}) in the milk 48 hours after inoculation.

TABLE I.

Results of Determination of Free Lactic Acid in Sour Milk by Measurement of C_H and Application of Mass Law.

Time of sampling after inoculation.	Total acidity 0.1 N NaOH required to neutralize 100 cc. of milk.	Increase of acidity due to lactic acid.	Hydrogen ion concentration expressed as		0.1 N free lactic acid in 100 cc. of milk.	0.1 N lactic acid by partial ether extraction.
			pH	C_H		
(1)	(2)	(3)	(4)	(5)	(6)	(7)
hrs.	*cc.*	*cc.*			*cc.*	*cc.*
0	18	—	6.50	0.032×10^{-5}	—	—
15	19	1.0	6.43	0.037 "	—	—
16	20.2	2.2	6.40	0.040 "	—	—
17	21.5	3.5	6.31	0.049 "	0.007	—
18	23.3	5.3	6.21	0.062 "	0.01	—
19	27.2	9.2	5.91	0.123 "	0.05	—
20	33.0	15.0	5.75	0.180 "	0.10	—
$20\frac{1}{2}$	36.8	18.8	5.58	0.265 "	0.20	—
21	41.7	23.7	5.46	0.35 "	0.33	—
$21\frac{1}{2}$	48.2	30.2	5.33	0.47 "	0.56	—
22	54.2	36.2	5.18	0.66 "	0.90	—
$22\frac{1}{2}$	63.6	45.6	4.98	1.05 "	1.84	2.0
23	73.8	55.8	4.73	1.90 "	3.95	3.7
$23\frac{1}{2}$	78.2	60.2	4.73	1.90 "	4.25	4.2
24	82.0	64.0	4.57	2.70 "	6.24	6.0
$24\frac{1}{2}$	82.3	64.3	4.56	2.80 "	6.50	6.3
$29\frac{1}{2}$	101.5	83.5	4.32	4.80 "	13.40	14.0
48	115.3	97.3	4.17	6.80 "	20.80	22.0

3. The amount of 0.1 N free lactic acid in 100 cc. of milk, calculated on the basis of the measurement of C_H and titration values and the application of the mass law, varies from 0.01 cc. at 18 hours to 20.8 cc. 48 hours after inoculation.

4. Comparing the results given in Column 3 with those in Column 6, we observe a wide difference between the total amount of acid formed in the milk during souring and the amount of lactic acid existing free. During the first 20 hours, the amount of free lactic acid present is very small, since that which forms combines at once with the basic constituents of the milk. At $20\frac{1}{2}$ hours after inoculation, under the conditions of our experiments, about 1 per cent of the total acid in the milk, shown by titration, exists as free lactic acid. This percentage increases to 4 per cent of the total in $22\frac{1}{2}$ hours, 7 per cent in $23\frac{1}{2}$ hours, nearly 10 per cent in 24 hours, 16 per cent in $29\frac{1}{2}$ hours, and 22 per cent in 48 hours.

II. Partial Extraction by Ether and Application of the Coefficient of Distribution.

Lactic acid, when dissolved in water, can be extracted in part by ether. When a water solution of lactic acid is treated with ether and the mixture shaken until the ether dissolves all of the acid that it can, the concentration of acid (a) in the ether layer, divided by the concentration of acid (b) remaining in the water layer, gives a resulting factor (k), which is constant at any one temperature for any solution of lactic acid, without regard to the relative volumes of the ether and water. In mixtures containing only lactic acid and water, this constant is approximately 0.08. These relations are expressed by the equation, $\frac{a}{b} = k$.

When, however, other substances are also present in solution, they may change the ratio. Therefore, in order to make use of this method in determining the amount of free lactic acid in sour milk, it is necessary to ascertain the effect of the compounds dissolved in milk upon the value of k. It is impossible to do this directly because of the neutralization of lactic acid by the calcium caseinate and basic phosphates and citrates in souring milk. For experimental work, a solution was prepared, consisting of a mixture of free lactic acid with chlorides of calcium, sodium, potassium, and magnesium in concentrations approximating, for the bases, the amounts present in average normal milk. In such a solution the value of k becomes 0.09 approximately. This figure, however, fails to take into consideration the effect of phosphates, citrates, or milk proteins on the value of k. The effect of casein could not be measured, but free phosphoric acid and citric acid were added to the mixture of lactic acid and chlorides, and the resulting mixture was partially extracted with ether. Under these conditions, the value of k becomes 0.095. The results of these experiments indicate that neither the salts nor acids used have a marked effect upon the coefficient of distribution of lactic acid between water and ether. Therefore, while this value of k (0.095) may not be regarded as being the strictly true value, it is sufficiently accurate to make this method of value in approximately measuring the amount of free lactic acid existing in sour milk, especially when taken in connection with the other methods used by us.

EXPERIMENTAL.

In applying this method, one puts 50 cc. of the milk under examination in each of two 100 cc. centrifuge tubes and adds to each about 50 cc. of acid-free ether. The tubes are closed with stoppers, leaving an air-space above the ether. The mixture is gently shaken at intervals, care being taken to avoid such vigorous shaking as would emulsify the mixture to such an extent as to prevent the ether later from being separated more than one-half by centrifuging. It requires about 15 minutes to complete the operation and bring the two layers into equilibrium. The mixture is then centrifuged until 25 cc. or more of the ether in solution separates as a clear layer so completely that it can be withdrawn. The clear ether from both tubes is then mixed, being poured off into a graduated cylinder, care being taken that only clear ether, unmixed with water or emulsion, be poured out. The total volume of ether is read and the ether is then transferred to the vessel in which titration is to be made. Water (about 25 cc.) is added and the ether evaporated, care being taken that the water solution does not boil or evaporate. The solution is then cooled and titrated with 0.05 or 0.02 N NaOH, free from carbonate, or with 0.02 N $Ba(OH)_2$, using phenolphthalein as indicator. The amount of free lactic acid present in the milk is calculated from the following equation (derived from the equation given above, $\frac{a}{b} = 0.095$):

$$\frac{\text{cc. of 0.1 N alkali used}}{\text{cc. of ether decanted}} \times 1{,}050 = \text{cc. of 0.1 N NaOH required to neutralize the lactic acid in 100 cc. of milk.}$$

The figures in Table II give the results of determinations made by this method in the case of pasteurized milk inoculated with *Bacterium lactis acidi*.

The results were checked by measurement of C_H and application of the mass-law method previously described and were found to be in good agreement. In Table I results are given in the last column, showing the figures obtained by the two methods. The ether-extraction method gives reliable results, however, only when there is no ether-soluble acid present other than lactic acid. Such a condition exists when the only organisms present are *Bacterium lactis acidi* and *Bacillus bulgaricus;* but in milk

soured in the ordinary way there may be at times some acetic acid.[9] This is illustrated by an experiment in which milk was inoculated with an ordinary "starter" and determinations of acid were made by ether extraction with the following results.

TABLE II.

Results of Determination of Free Lactic Acid in Milk by Extraction with Ether and Use of Coefficient of Distribution.

Time examined after inoculation.	0.1 N NaOH required to neutralize 100 cc. of milk.	Ether extract.	0.02 N NaOH required to neutralize free lactic acid in ether extract.	0.1 N NaOH required to neutralize free lactic acid in 100 cc. of milk.
hrs.	*cc.*	*cc.*	*cc.*	*cc.*
Fresh.	17.0	—	—	—
20	51.2	60	0.8	2.8
21	64.0	44	1.0	4.8
22	70.0	54	2.5	9.7
48	98.0	58	6.4	23.2
96	99.0	52	5.9	23.8

By comparing the results in the last column of Table III with those in the last column of Table II, it is seen that after 23 hours, the figures in Table III are much in excess of those in Table II. This was found to be due to the presence of a volatile acid, prob-

TABLE III.

Results of Ether Extraction Method in Ordinary Sour Milk.

Time examined after inoculation.	0.1 N NaOH required to neutralize 100 cc. of milk.	Ether extract.	0.02 N NaOH required to neutralize free acid in ether extract.	0.1 N NaOH required to neutralize free acid in 100 cc. of milk.
hrs.	*cc.*	*cc.*	*cc.*	*cc.*
22	50	55	0.6	2.3
23	61	52	1.0	4.0
28	80	48	4.4	19.3
48	90	53	10.2	40.4
96	95	60	12.5	43.7

ably acetic. The figures obtained after 23 hours in Table III fail, moreover, to agree satisfactorily with results obtained by calculations based on measurement of C_H and application of the mass-law equation.

[9] Bosworth, A. W., and Prucha, M. J., *N. Y. Agric. Exp. Station, Technical Bull. 14*, 1910.

III. Double Electrometric Titration with Lactic and Hydrochloric Acids.

In order to test the accuracy of the results obtained by the methods already described in determining the amount of free lactic acid in sour milk, it appeared desirable to make use of the method of electrometric titration described in another article[10] applied to the same milk used in previous experiments, employing lactic and hydrochloric acids.

The use of the double electrometric titration of milk with lactic acid and hydrochloric acid in ascertaining the amount of free lactic acid is based upon the following considerations: If, working within the limits of the acidity of sour milk, we run each of these acids from burettes into separate portions of a milk until the same C_H of each is reached, we find on examining the burette readings that a larger amount of lactic acid has been used than of hydrochloric. This difference represents difference in ionization in the two acids. Since lactic acid is ionized only in small part, while hydrochloric acid is practically all ionized, it requires larger amounts of lactic acid to produce the same C_H; or, stated in another way, since the lactic is mostly un-ionized and relatively none of the hydrochloric acid is un-ionized, the difference between the amounts of lactic acid and hydrochloric acid required to produce the same C_H represents the amount of free lactic acid in the milk at the particular point of C_H. At the same C_H, the amount of base neutralized is the same for different acids and therefore the lactate and chloride in solution formed in milk on addition of the two acids are the same at the same C_H. It is believed also that no adsorption of these salts occurs to interfere with the accuracy of the determinations.

EXPERIMENTAL.

The electrometric titrations are made with N acid and 250 cc. of milk. The amounts of acid used (expressed as cc. of 0.1 N per 100 cc. of milk) are given in Column 1 of Table IV, the purpose being to imitate the souring of milk by successive small additions of acid. After each addition of acid, the C_H is measured, care being taken to follow the instructions already given in the preceding article. The pH values obtained for the different

[10] Van Slyke and Baker, *J. Biol. Chem.*, 1918, xxxv, 137.

amounts of lactic acid and hydrochloric acid taken are used in preparing the chart shown in Fig. 1, which is based on the values given in Table IV. By interpolation, the results of titration can be found at equal pH values. Therefore, by inspection of this chart, we can ascertain the amount of free lactic acid at any pH

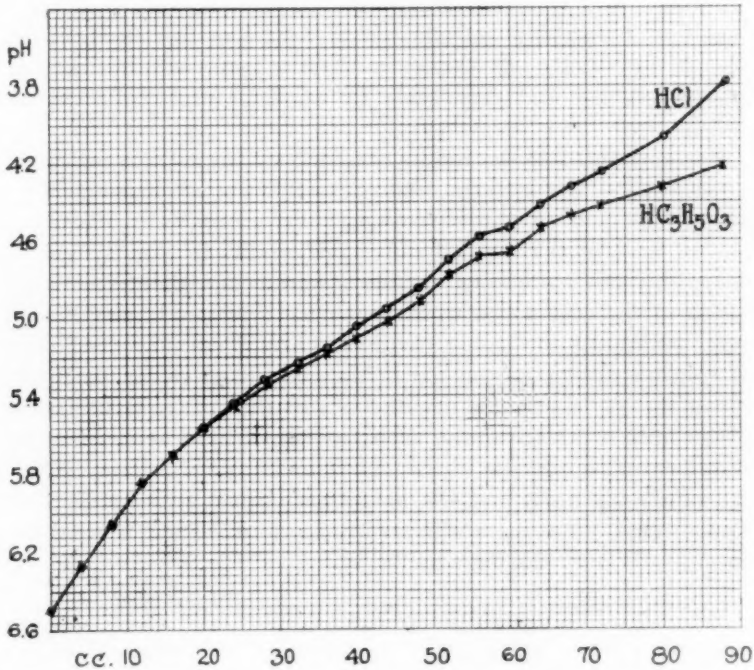

FIG. 1. This chart based on data in Table IV, enables one to find the amount of free lactic acid (0.1 N acid per 100 cc. of milk) at any point of hydrogen ion concentration expressed in terms of pH. This is done by measuring the horizontal distances apart shown by the lactic acid and hydrochloric acid curves at any one pH value.

point by the horizontal distances between the lactic acid and hydrochloric acid curves. It is noticeable that the curves for both acids coincide until we have added more than 24 cc. of 0.1 N acid per 100 cc. of milk, after which there is a divergence in values and this increases gradually.

The irregularity or flattening in the curves is noticeable when between 50 and 60 cc. of 0.1 N acid have been added. This is due to interference as a result of the coagulation of the casein by acid.

In Table IV, we give in the last three columns the results obtained by the three different methods we have been discussing for the determination of free lactic acid in sour milk; *viz.*, (1) by application of mass-law calculations to results of measurement of C_H, (2) by double electrometric titration, and (3) by partial ether extraction and calculation by application of coefficient of distribution.

The agreement of the results obtained by these three methods is reasonably close until the coagulation point of casein is reached, after which it is not possible to secure uniformity in the distribution of acid in the curdled milk; this results in slightly lower values by the method of double electrometric titration. The action of the acid added to curdled milk is limited largely to the outer surface of the casein particles. Any di-calcium phosphate or calcium caseinate occluded in the coagulated casein particles is reached only slowly and incompletely by the process of diffusion. When milk sours naturally, each particle of curd or coagulated casein contains distributed through it millions of bacteria, which are producing acid; and the acid thus formed reaches the occluded basic salts more effectively, thus producing a reaction more nearly approaching equilibrium.

Adsorption of Lactic Acid by Casein in Sour Milk.

Casein adsorbs lactic acid.[11] If free lactic acid is adsorbed by casein in sour milk, it is removed from the liquid phase and is, therefore, not determined by any of the methods previously described in this article. It seemed necessary, on this account, to ascertain how much lactic acid is adsorbed by casein under the conditions existing in sour milk and then make such correction as might be found to be needed, in order to learn the total amount of free lactic acid present in sour milk. Such a correction is based upon the assumption that adsorbed acid is removed as such in the molecular condition, an assumption justified by results to be presented later.

[11] Van Slyke and Van Slyke, *Am. Chem. J.*, 1907, xxxviii, 383.

For a discussion of the characteristics of adsorption phenomena, the reader is referred to the above cited article of Van Slyke and Van Slyke.

The amount of lactic acid adsorbed by casein in sour milk has been determined by us by four different methods, as follows:

TABLE IV.

Results of Determination of Free Lactic Acid by Method of Double Electrometric Titration.

0.1 N acid used for 100 cc. of milk.	Hydrogen ion concentration of lactic acid added to milk expressed as		Hydrogen ion concentration of HCl added to milk expressed as		0.1 N free lactic acid in sour milk as determined by method of		
	pH	C_H	pH	C_H	Mass-law calculation.	Double electrometric titration.	Ether extraction.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
cc.					cc.	cc.	cc.
0	6.50	0.032×10^{-5}	6.50	0.032×10^{-5}			
4	6.28	0.053 "	6.28	0.053 "			
8	6.05	0.090 "	6.05	0.090 "			
12	5.84	0.146 "	5.85	0.143 "			
16	5.71	0.196 "	5.71	0.196 "			
20	5.56	0.280 "	5.56	0.280 "			
24	5.45	0.36 "	5.44	0.367 "			
28	5.35	0.45 "	5.32	0.48 "			
32	5.26	0.55 "	5.24	0.58 "	0.7	1.0	—
36	5.19	0.65 "	5.15	0.71 "	0.9	1.3	—
40	5.10	0.80 "	5.05	0.90 "	1.2	1.8	—
44	5.02	0.96 "	4.97	1.08 "	1.7	2.4	2.3
48	4.91	1.23 "	4.84	1.46 "	2.3	2.7	2.9
52	4.78	1.67 "	4.71	1.96 "	3.2	3.0	3.0
56	4.68	2.10 "	4.59	2.58 "	4.3	4.0	3.8
60	4.65	2.26 "	4.53	2.98 "	4.9	6.8	5.0
64	4.54	2.91 "	4.42	3.84 "	6.6	5.0*	7.0
68	4.47	3.43 "	4.32	4.80 "	8.1	6.0*	8.0
72	4.42	3.84 "	4.20	6.32 "	9.6	8.0*	10.0
80	4.32	4.80 "	3.97	10.80 "	13.0	12.0*	13.0
88	4.21	6.19 "	3.78	16.70 "	17.5	16.0*	19.0

* Results lower on account of coagulation of casein in milk by acid.

(1) By adding definite amounts of casein to solutions containing given amounts of lactic acid and then determining the extent of reduction of the hydrogen ion concentration. (2) By the deter-

mination of the acidity of the filtrate after removal of casein under the same conditions as in the preceding method. (3) By the determination of the amount of acid retained by casein when separated from the whey in sour milk. The entire sour milk is titrated and also the separated whey, and the difference noted, correction being made for the known acidity of the casein. (4) By extraction of the lactic acid from sour milk and also from the separated whey, the difference between the two results being the amount of acid adsorbed.

I. Adsorption Measured by Reduction of Hydrogen Ion Concentration.

In the first two methods mentioned, the work is carried out with prepared solutions of lactic acid and calcium lactate, of known concentrations, to which given amounts of casein are added. These solutions are made of such concentrations as are known to exist in average sour milk; that is, 3 per cent of casein and 0.065 normal of calcium lactate[12] and amounts of lactic acid varying according to the degree of acidity desired. Lactic acid solution of approximately normal concentration was boiled in a reflux condenser 48 hours in order to remove anhydride and this acid was used in preparing the solutions of known concentrations.

1. Preparation of Solution.—Proceeding now to give the details of the method used, we prepare the solutions containing lactic

[12] The factor for calcium lactate, 0.065 normal, is obtained as follows: The free lactic acid in sour milk, as determined by application of the mass law (p. 148), or by the coefficient of distribution (p. 153), is subtracted from the total amount of acid in each case, as determined by titration, and the remainder, in case of milk soured by pure cultures of *Bacterium lactis acidi*, is assumed to be neutralized lactic acid, which is present in sour milk in combination chiefly as calcium lactate. Values for neutralized lactic acid (calcium lactate) obtained by this method, in case of several curdled milks, vary from 0.05 to 0.075, with an average of about 0.065 normal. Variations under the conditions found in sour milk in the amount of calcium lactate are not found to affect the proportion or percentage of the lactic acid that is adsorbed in case of equal hydrogen ion concentrations. While some lactate ion may be derived from the ionized lactic acid in a mixture of free lactic acid and calcium lactate, the amount is so small as to be negligible in comparison with that from the added calcium lactate.

acid and calcium lactate in 100 cc. portions, the concentration being made twice that desired for the final concentration. This is divided into two portions of 50 cc. each, and the casein is added in the form of a 6 per cent suspension to one portion, after which the volume of each is brought to 100 cc.

2. Preparation of Casein.—The casein used in these adsorption experiments is prepared by the method described by us in another paper[7] with the single variation that the washing with alcohol and ether is omitted. The casein is made into a water suspension, containing about 7 per cent of casein, by vigorous beating after the fifth washing with water. Prepared in this manner, the casein remains a long time in persistent suspension. A portion (10 cc.) of this suspension is titrated to phenolphthalein with an excess of 0.1 N NaOH and the solution is titrated back to the neutral point with 0.1 N HCl. The amount of casein in the mixture is calculated on the basis of taking 1 gm. of casein as equivalent to 9 cc. of 0.1 N NaOH. If the casein in the suspension is beaten into fine enough particles, the titration gives an end-point reading that is accurate to about 0.2 cc. in a total titration of about 7 cc. Using the result thus obtained, the suspension is diluted so that there will be 6 gm. of casein in 50 cc. of the suspension.

3. Preparation of Mixture of Casein and Solution.—In preparing mixtures, in which we wish to measure the amount of decrease of hydrogen ion concentration, when casein is added to a solution containing lactic acid and calcium lactate, we mix 50 cc. of the 6 per cent casein suspension at 25°C. with 50 cc. of each of the acid solutions at 25°C.; the different amounts of acid used are stated in Table V. The amount of casein in the mixtures thus prepared is 3 per cent, accurate within 0.1 per cent.

4. Determination of Hydrogen Ion Concentration.—After the mixture of casein and acid is completed, the C_H determination can be made at any time after 30 minutes, because the adsorption is complete within 30 minutes at 25°C. The E. M. F. of the solution without casein is first determined in duplicate after standing 24 hours. If the duplicates agree, the E. M. F. of the solution of the same concentration containing casein is determined, using the same electrode. (It is desirable to use freshly platinized electrodes for each set of two determinations.)

5. Calculation of Results.—From the procedure described, we obtain the following data: (*a*) The amount of lactic acid and calcium lactate used and the hydrogen ion concentration (C_H); (*b*) the C_H of lactic acid after the addition of casein, and from this latter figure we can obtain (*c*) the amount of lactic acid in the solution after the addition of casein. We can calculate the per cent of acid in the original solution that is adsorbed by the casein by using the amount of acid in solution, as obtained by calculation based on the hydrogen ion normal. We subtract the figure representing the acid in solution after addition of casein from the figure representing the acid in the solution before addition of casein, and divide the result by the latter figure; or expressed as an equation, we have, $\frac{a-b}{a}$ = per cent of acid adsorbed; in which a is the amount of lactic acid used and b is the lactic acid in solution after adding casein.

The results of our work are given in Table V. In Column 1 is given the amount of lactic acid used and in Column 2 the amount of acid present after the addition of casein, as determined by the method of calculation described above. In Columns 3 and 4 are given the pH values of the lactic acid present before and after adding casein, as found by actual measurement, while in Columns 5 and 6 are given the hydrogen ion normal values corresponding to the pH values given in Columns 3 and 4. In Column 7 are given the percentages of the used lactic acid that is adsorbed by casein, which is present in suspension to the extent of 3 per cent. The values in Column 7 are obtained by the method of calculation already described.

By comparing the figures in Table V, Column 3, with those in Table III, Column 4, it is seen that the range of real acidity within which we have studied the question of adsorption is considerably wider than that found in sour milk.

In studying the results in Table V, those in Column 7 attract our special interest. According to these results, no lactic acid is adsorbed by casein under the conditions of our experiments until the casein is added to a solution which has a pH value of 4.51. From that point on to higher degrees of acidity, adsorption increases with increase of lactic acid.

The percentages of lactic acid adsorbed at different concentrations of hydrogen ion have been plotted (Fig. 2). By means of this, one can approximately ascertain the percentage of acid adsorbed in any given sour milk, provided the hydrogen ion concentration of such milk is known.

There is an apparent disagreement between the results in Table V and those published by Van Slyke and Van Slyke,[13] who found

TABLE V.

Adsorption of Lactic Acid by Casein, Based on C_H Values.

0.1 N lactic acid in 100 cc. of solution.		Lactic acid in solution, expressed as pH.		Lactic acid in solution, expressed as C_H.		Proportion of free lactic acid in milk adsorbed by casein.
Without casein.	With casein.	Without casein.	With casein.	Without casein.	With casein.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
cc.	cc.					per cent
5	5.3	4.67	4.65	0.21×10^{-4}	0.22×10^{-4}	0
5.5	5.7	4.65	4.64	0.22 "	0.23 "	0
6	6.2	4.62	4.60	0.24 "	0.25 "	0
6.5	6.5	4.58	4.59	0.26 "	0.26 "	0
7	7.0	4.57	4.57	0.27 "	0.27 "	0
8	7.5	4.51	4.55	0.31 "	0.29 "	6
9	8.2	4.44	4.48	0.36 "	0.33 "	9
20	15.4	4.12	4.23	0.76 "	0.59 "	22
30	22.0	3.89	4.02	1.30 "	0.96 "	27
40	28.0	3.77	3.93	1.70 "	1.20 "	30

under the conditions of their experiments that some lactic acid is always adsorbed even at low concentrations, while our results, on the contrary, show no adsorption when we use not more than 7 cc. of 0.1 N lactic acid per 100 cc. of solution. There is, however, no real disagreement when the difference of conditions in experiments is taken into consideration. In their work, lactic acid and casein only were used, while in our work there is present, in addition, calcium lactate, in order to imitate the conditions in sour milk. The presence of calcium lactate greatly reduces the ionization of the lactic acid, so that the amount of ionized lactic acid is less in our experiments, even though the amount of free acid is greater. To such an extent is this true that

[13] Van Slyke and Van Slyke, *Am. Chem. J.*, 1907, xxxviii, 435.

in the solutions containing 7 cc., and less, of added 0.1 N acid per 100 cc. of solution, the hydrogen ion concentration is below the isoelectric point of casein, which equals C_H, 0.27×10^{-4}, a value in close agreement with that (0.24 to 0.26×10^{-4}) found

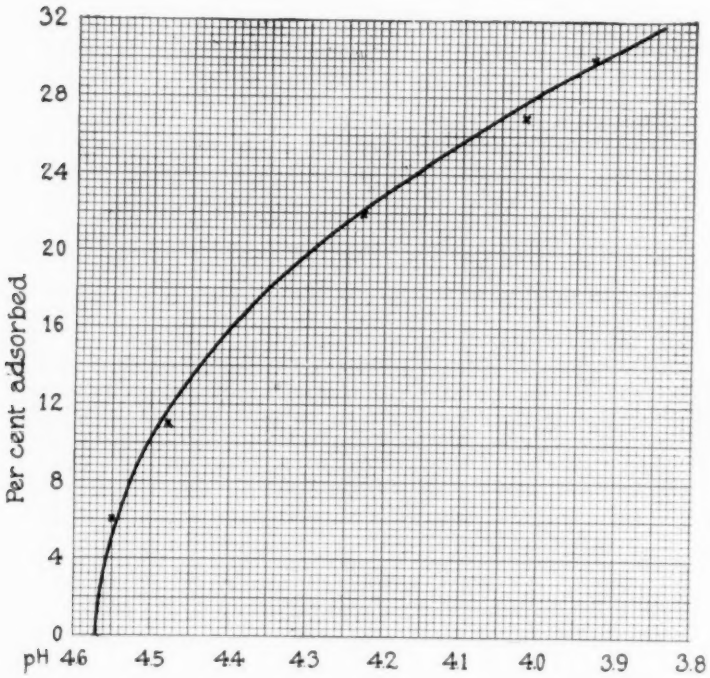

FIG. 2. This chart shows the approximate percentage of the free lactic acid in sour milk that is adsorbed by 3 per cent of casein at different degrees of hydrogen ion concentration expressed in terms of pH.

by Michaelis.[14] By isoelectric point, we mean that hydrogen ion concentration at which the positive and negative charges on colloidal particles are equal. At this point casein is combined with neither base nor acid. When pure casein is added to a solution having the same reaction, it is found, as seen in Table V, Columns 1 and 2, that the C_H remains unchanged and, there-

[14] Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1912, xlvii, 260.

fore, no acid is removed by casein through adsorption. When the C_H is below 0.27×10^{-4}, then casein and calcium lactate undergo reaction, calcium combining with casein to form calcium caseinate with simultaneous formation of free lactic acid. Under these conditions, it is not likely that casein could adsorb lactic acid at the same time that it is combining with calcium. At the isoelectric point casein combines with neither acid nor base. If adsorption occurred below C_H, 0.27×10^{-4}, the resulting solution would have a lower C_H, which is contrary to our results.

II. Adsorption Measured by Results of Titration.

In obtaining the titration values for determining the percentage of acid adsorbed by casein, we prepare, as in the case of the preceding method, known solutions of lactic acid and calcium lactate and add to each of these enough casein in suspension to make the mixture contain 3 per cent. The mixture of acid solution and casein is shaken vigorously for a short time and then filtered, the first few cc. of filtrate being rejected. Then a portion of 10 cc. is titrated to phenolphthalein with 0.01 N NaOH. The amount of acid in the original solution, less the amount of acid shown by titration of the filtrate, divided by the amount of acid in the original solution gives the percentage of the acid used that is adsorbed by the casein.

However, the titration values need to have a correction applied in the form of a reduction of 0.6 cc. If we compare the amounts of lactic acid used with the amounts found after the addition of casein, we find that the amounts of acid, as determined by titration, are considerably higher after addition of casein than the amounts originally used and also higher than the amounts found by measurement of the hydrogen ion concentration. This is obvious if we examine the first three columns in Table VI. Thus, we notice that when we add casein to a solution containing 7 cc. of 0.1 N lactic acid per 100 cc., the value obtained by the determination of C_H shows the same amount of acid, which means that no adsorption takes place at that point of acidity. But the titration value at the same point gives 7.6 cc. of 0.1 N lactic acid, or an excess of 0.6 cc. over the amount with which we started. This amount of excess deducted from the titration values in Column 3, Table VI, gives the results found in Column 4.

The cause of the discrepancy which requires the correction in the titration results may be found to be due to one or both of two reactions which affect the titration. (1) There may be a reaction between the calcium lactate and casein, resulting in the formation of insoluble calcium caseinate and free lactic acid, which would increase the titration results. (2) There is dissolved casein present in the filtrate, which would increase the titration somewhat. The amounts of casein in the filtrate are given in Column 7 of Table VI. The determinations of casein were made by colorimetric methods with standard solutions of

TABLE VI.

Adsorption of Lactic Acid by Casein, Based on Titration Results.

0.1 N lactic acid in 100 cc. of solution.			Corrected titration values.	Lactic acid adsorbed by casein, based on titration.	Lactic acid adsorbed by casein, based on C_H determination.	Casein in solution in filtrate.
Without casein.	With casein by C_H method.	With casein by titration.				
(1)	(2)	(3)	(4)	(5)	(6)	(7)
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*
5.0	5.3	6.2	5.6	0	0	0.0145
5.5	5.7	6.6	6.0	0	0	0.0155
6.0	6.2	7.0	6.4	0	0	0.0160
6.5	6.5	7.2	6.6	0	0	0.0165
7.0	7.0	7.6	7.0	0	0	0.0170
8.0	7.5	8.2	7.6	5	6	0.0175
9.0	8.2	8.8	8.2	9	9	0.0185
20.0	15.4	16.9	16.3	19	23	0.0250
30.0	22.0	24.7	24.1	20	27	0.0400

casein dissolved in nitric acid, the work of Van Slyke and Van Slyke[15] being used as a basis for the method, in connection with a Duboscq colorimeter.

The chief interest in the results in Table VI lies in Columns 5 and 6. We find that the percentage of lactic acid adsorbed by casein agrees closely by the two methods within the range of acidity commonly found in sour milk.

III. Adsorption Measured by Titration Acidity in Sour Milk and Whey.

It is essential to know whether the results obtained in the preceding experiments are applicable to the conditions existing in

[15] Van Slyke and Van Slyke, *Am. Chem. J.*, 1907, xxxviii, 406.

sour milk. In order to ascertain this, special experiments have been made with sour milks. If we determine the acidity of whole or unseparated curdled milk and then of its separated whey, we find the acidity of the whey less than that of the whole sour milk. The difference is due to the free casein which is present in the whole curdled milk but absent from the whey. A part of this difference of acidity is due directly to the neutralizing or base-combining power of free casein, 1 gm. of casein requiring for neutralization to phenolphthalein 9 cc. of 0.1 N alkali. The other and smaller portion of decrease of acidity is due to adsorption of acid by casein. For example, 100 cc. of a whole, curdled milk requires 100 cc. of 0.1 N NaOH to neutralize it to phenolphthalein. When the casein is removed and the whey neutralized, it is found to require only 72 cc., a difference of 28 cc. of 0.1 N alkali. This milk contains 2.8 per cent of casein, which requires 25.2 cc. (9 cc. $\times$ 2.8) of 0.1 N alkali to neutralize it. Of the total 28 cc. decrease of acidity between the whole sour milk and the whey, 25.2 cc. are thus accounted for by the base-combining power of the casein, and this leaves 2.8 cc. to be accounted for as adsorbed by casein. On the basis of these statements, the amount of adsorption of acid by casein can be ascertained in the case of sour milk by subtracting from the total acidity of the milk the acidity of the whey and of the casein in the milk.

We have completed experiments with four different sour milks in the line indicated by the foregoing statements.

Milk, in which the percentage of casein is determined, is treated with a pure culture of *Bacterium lactis acidi* and allowed to stand at 25°C. until curdled. A portion of the curdled milk is titrated to phenolphthalein with 0.1 N NaOH by the method already described (p. 150) care being taken to dissolve the casein completely. Then a known amount of the curdled milk is whirled in a centrifugal test-tube until the whey separates in a clear, supernatant layer above the solid layer of casein. A given amount of the separated whey is titrated to phenolphthalein with 0.1 N NaOH. The results of these experiments are given in Tables VII and VIII. The results in the last column of Table VII are obtained by adding the figures in Columns 3 and 6 in each experiment and subtracting the sum from the figures in Column 5.

TABLE VII.

Adsorption of Lactic Acid by Casein in Sour Milk. Results Based on Titration.

No. of experiment.	Casein in milk.	Acidity of casein expressed as 0.1 N NaOH.	Milk analyzed after inoculation.	Amount of 0.1 N NaOH required to neutralize		Amount of 0.1 N lactic acid adsorbed by casein for 100 cc. of sour milk.
				100 cc. of whole sour milk.	Whey from 100 cc. of sour milk.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	per cent	*cc.*	*hrs.*	*cc.*	*cc.*	*cc.*
1	2.70	24.3	48	106	78.0	**3.7**
2	2.80	25.2	48	99	70.8	**3.0**
3	2.79	25.1	72	100	72.2	**2.7**
4	2.87	25.8	96	107	78.0	**3.2**

The data in Table VII enable us to find the amount of lactic acid adsorbed by casein for 100 cc. of sour milk. In order to find what percentage of the free lactic acid present in sour milk is adsorbed, we must know also the amount of this free lactic acid,

TABLE VIII.

Free Lactic Acid in Sour Milk Used in Experiments.

No. of experiment.	Total acidity. 0.1 N NaOH required to neutralize 100 cc. of sour milk.	Acidity of fresh milk. 0.1 N required to neutralize 100 cc. of fresh milk.	Total lactic acid formed in milk. Total acidity less acidity in fresh milk.	Hydrogen ion concentration expressed as pH.	Equivalent of pH value expressed as C_H.	0.1 N free lactic acid in 100 cc. of sour milk.
	cc.	*cc.*	*cc.*			*cc.*
1	106	16.5	89.5	4.25	5.6×10^{-5}	**16.4**
2	99	18.0	81.0	4.27	5.4 "	**14.4**
3	100	18.0	82.0	4.37	4.3 "	**12.0**
4	107	19.0	88.0	4.33	4.7 "	**14.0**

and this is obtained from the data in Table VIII by application of the mass-law calculation (p. 148 and Table III, p. 155).

From the figures in the last columns of Tables VII and VIII, we can calculate the percentage of free lactic acid adsorbed by the casein in 100 cc. of sour milk. We obtain the following values:

No. of experiment.	Free lactic acid in 100 cc. of sour milk.	Lactic acid adsorbed.	Percentage of lactic acid in sour milk adsorbed.
	cc.	*cc.*	
1	16.4	3.7	23
2	14.4	3.0	21
3	12.0	2.7	22
4	14.0	3.2	23

If we use the pH values in Table VIII in connection with the adsorption curve (p. 164), we obtain the following figures for the percentage of lactic acid adsorbed by casein in sour milk.

Experiment No.	Percentage by adsorption curve.	Percentage by titration.
1	22	23
2	21	21
3	18	22
4	18	23

It may be stated in connection with the method of measuring adsorption by means of titration values, as described above, that we appreciate that it is open to some serious objections. All errors in titrations fall upon the value obtained for adsorption and these may be considerable unless one is accustomed to the method and performs the work with care. The amount of adsorption is small and errors in work are therefore of relative prominence. Its value lies mainly in affording an independent or additional means of measuring adsorption in connection with other methods.

IV. Adsorption Measured by Extraction of Lactic Acid in Sour Milk and Whey.

A measured portion of sour milk is extracted about 48 hours by hot ether in a continuous extractor, enough phosphoric acid being added to convert all lactates into free lactic acid. A given portion of whey, obtained in the manner already described (p. 167) is extracted in the same way. We thus obtain the total lactic acid formed in the milk during the process of souring and also the total in the whey. The difference represents directly the amount adsorbed by the casein in the milk. This method is applicable in case of milk soured by such pure cultures as are

TABLE IX.

Adsorption of Lactic Acid Measured by Extraction of Sour Milk and Whey.

No. of experiment.	Amount of 0.1 N lactic acid in 100 cc. of sour milk.	Amount of 0.1 N lactic acid in whey for 100 cc. of sour milk.	Amount of 0.1 N lactic acid adsorbed by casein in 100 cc. of sour milk.	Amount of 0.1 N free lactic acid in 100 cc. of sour milk.	Percentage of lactic acid in sour milk adsorbed by casein.
	cc.	cc.	cc.	cc.	
1	90.1	85.8	4.3	16.4	26
2	81.2	77.7	3.5	14.4	24
3	84.0	81.5	2.5	12.0	21

known to produce only lactic acid. The method was applied to the milks numbered 1, 2, and 3 used in the preceding experiments.

A comparison of the results obtained by the four methods employed are in approximate agreement in showing that in sour milk about 20 to 25 per cent of the entire free lactic acid present in the milk is adsorbed by casein. When we consider the total amount of lactic acid (free and combined as lactates) that is formed in sour milk, the percentage adsorbed amounts in most cases to only 2.5 to 3.5 per cent approximately.

In accordance with our results, it is sufficiently accurate to make an allowance of about 20 per cent of the free lactic acid in sour milk as the amount adsorbed by casein. Therefore, in estimating the total amount of free lactic acid in sour milk, we determine the amount of free lactic acid in solution by the method based on the C_H value (p. 148) and to this result add 20 per cent of itself.

In this connection the question may be raised as to whether other acid constituents of milk, such as acid phosphate and citrate salts, are not also adsorbed by casein. Some special experiments were made to test this point. Solutions with hydrogen ion concentration approximately equal to that of sour milk were made, in all of which there was the same concentration of lactic acid but varying amounts of other constituents. Some contained only lactic acid and calcium lactate, while others contained also acid citrate or acid phosphate in the approximate portions present in milk. To each of these different solutions casein suspension was added to the extent of 3 per cent in the manner previously described (p. 161) and the reduction of hydrogen ion concentration was measured. In all cases the results showed no greater removal of acid by casein from those solutions containing acid citrate and phosphate than from the one con-

taining only lactic acid. Similar experiments in which acetic acid was present gave only slightly increased adsorption of acid. The results with acid citrate and phosphate solutions are in harmony with the fact that all the citrate and phosphate in sour milk are found in the serum.[16]

TABLE X.

Acidity of Milk Soured under Different Conditions.

No. of sample.	Days analyzed after inoculation.	Hydrogen ion concentration expressed as		0.1 N free lactic acid in 100 cc. of milk.	0.1 N free lactic acid adsorbed by casein in 100 cc. of milk.	0.1 N total free lactic acid in 100 cc. of milk.	0.1 N lactic acid in form of lactate in 100 cc. of milk.	0.1 N total lactic acid formed in 100 cc. of sour milk.
		pH	C_H					
				cc.	cc.	cc.	cc.	cc.
1	2	4.17	6.8×10^{-5}	21.0	4.8	25.9	71.4	97.3
2	2	4.03	7.2 "	26.5	7.6	33.7	64.3	98.0
3	3	4.34	4.6 "	14.2	2.4	16.6	74.4	91.0
4	6	4.56	2.9 "	8.3	3.0	8.6	71.4	80.0
5	4	3.86	14.0 "	64.0	23.0	87.0	92.0	179.0
6	10	3.70	20.0 "	98.0	36.3	134.0	80.0	220.0
7	3¼	4.14	7.2 "	21.0	5.0	26.0	67.5	93.5
8	2	4.02	9.5 "	27.2	7.3	34.5	64.5	99.0
9	2½	4.37	4.3 "	14.7	2.3	17.0	83.0	100.0
10	2¾	4.35	4.5 "	16.4	2.8	19.2	88.3	107.5
11	3	4.32	4.8 "	16.1	2.9	19.0	81.0	100.0
12	3	4.43	3.7 "	11.6	1.5	13.1	76.9	90.0
13	1	4.36	4.4 "	14.4	2.3	16.7	79.3	96.0
14	1	4.36	4.4 "	13.6	2.2	15.8	75.2	91.0
15	3	4.22	6.0 "	20.0	4.4	24.4	77.6	102.0
16	3	4.15	7.1 "	15.0	3.7	18.7	51.8	70.5

Acidity of Sour Milks.

In connection with the work already described, it is a matter of interest to know something more in relation to the amount of acid produced in milk when soured under different conditions. We therefore present additional data obtained from a study of sixteen samples of sour milk. In all cases the milk was kept at 25°C. during the process of souring. In Samples 1 to 3, the souring was effected by *Bacterium lactis acidi* inoculated in pasteurized milk; in Sample 4, by *Streptococcus lacticus;* in Samples 5 and 6, by *Bacillus bulgaricus;* in Samples 7 to 12, by natural

[16] Van Slyke and Bosworth, *J. Biol. Chem.*, 1916, xxiv, 193.

souring, that is, by using unpasteurized milk which was self-inoculated by lactic organisms getting into the milk during milking and subsequent handling; in Samples 13 to 15, by inoculating unpasteurized milk with a more or less impure lactic culture or so-called starter of sour milk. Sample 16 is the same as 15 but is diluted with one-half its volume of water, enough lactose being added to make the amount of lactose the same as in the undiluted milk.

The interval of time after inoculation before analysis varied from 1 to 10 days. The determinations made were (1) hydrogen ion concentration; (2) the amount of free lactic acid; including that adsorbed, obtained by methods previously described in this article; (3) the total amount of lactic acid present in the form of lactate, obtained by difference between total and free acid. The detailed results are given in Table X. The examination of the sour milks was made at the stage of souring at which increase of acidity is very slow. In no case have we found increase of acid and C_H ceasing entirely within the limits of time covered by our work.

1. Hydrogen Ion Concentration of Sour Milks.—An examination of Table X indicates that there is no specific C_H value that is characteristic of sour milk. The results differ according to a variety of conditions, and especially with the kind of organism responsible for the souring and its rate of growth. In the case of milk that sours in the ordinary way (Samples 7 to 12), the pH value varies from 4.43 to 4.02, and with milks soured by "starter" (Samples 13 to 15), the variations are within similar limits of pH value, 4.36 to 4.22. In the case of pure cultures of *Bacterium lactis acidi* (Samples 1 to 3), the variations (pH 4.34 to 4.03) are within the same limits as observed in case of natural souring. This would, of course, be expected, since in natural souring or in the use of a sour-milk "starter," the organism chiefly or wholly responsible for the acidity is usually *Bacterium lactis acidi*. In the case of *Streptococcus lacticus*, a minimum acidity is observed (pH 4.56).

2. Total Amount of Acid Formed.—The total amount of acid formed in 100 cc. of sour milk is usually between 90 and 100 cc., expressed as 0.1 N acid, when the organism present is mainly *Bacterium lactis acidi*. In the case of *Bacillus bulgaricus*, the acidity may reach or exceed 200 cc. under the conditions of our work.

3. Free Lactic Acid.—The amount of free lactic acid, which includes that adsorbed, varies from less than 10 cc. of 0.1 N acid per 100 cc. of milk to 134. In the case of the usual lactic organisms, the variation lies between 13 and 34.5 cc. With *Bacillus bulgaricus* (Samples 5 and 6), the figures for adsorbed acid are not accurate owing to some solution of casein by the acid.

4. Relation of Total Acidity to Free Lactic Acid in Sour Milks.—If we compare the total amount of acid formed in sour milk with the amount of free lactic acid, we find that the percentage of total acid existing as free lactic acid varies widely. In the case of milk soured by a pure culture of *Bacterium lactis acidi* or by a sour-milk "starter" or by so-called natural souring, the percentage of total acid in the form of free lactic acid varies from 14.6 to 35, in the majority of cases being somewhere about 20.

5. Lactate in Sour Milk.—The amount of lactic acid present as lactate in sour milk is much more uniform, of course, than the free acid, since the amount of lactate in the presence of an excess of free acid is primarily dependent on the amount of basic compounds in each milk, and these do not usually vary widely in amount. In the samples of milk used in our experiments, the amount of lactate, equivalent to 0.1 N lactic acid, varies from 64.3 to 92 cc. per 100 cc. of milk.

6. Effect of Dilution of Milk on Acidity.—The effect of dilution by water is shown in Samples 15 and 16. In Experiment 15, normal milk was used, and in No. 16 the same milk diluted so as to contain one-third of its final volume of added water, enough lactose being added to make the percentage the same as in Sample 15. The effect of the dilution is to increase the hydrogen ion concentration, owing to the fact that there is less base to neutralize in the diluted milk. The degree of acidity is relatively greater in the diluted milk, if we take the amount of dilution into consideration. For example, the total acidity in the undiluted milk is equal to 102 cc. of 0.1 N acid in 100 cc. of milk, while in the diluted milk, the acidity is 70.5 cc., whereas it would be one-third less or 68 cc. if it were exactly in proportion to dilution.

Coagulation Point of Casein in the Souring of Milk.

Another point of interest in connection with milk soured at 25°C. is the relation of hydrogen ion concentration to the curd-

ling of milk; that is, the coagulation of casein. In ten samples of milk soured in the ordinary way, the hydrogen ion concentration and total acidity as determined by corrected titration (p. 149) are given. The determinations were made as nearly as possible at the time when the milk began to show the first signs of thickening; but in only four samples (Nos. 5 to 8) were we able to catch the thickening at the exact beginning. In these four samples the same determinations were repeated when the coagulation became complete; that is, when the milk formed a solid mass of curd. It is possible to catch the milk during this change only with considerable difficulty.

TABLE XI.

Relation of Hydrogen Ion Concentration to Coagulation of Casein.

No. of sample.	pH value coagulation.		C_H value coagulation.		0.1 N acid in 100 cc. of milk coagulation.		Time between beginning and completion of coagulation.
	Beginning or under way.	Completed.	Beginning or under way.	Completed.	Beginning or under way.	Completed.	
					cc.	*cc.*	*min.*
1	4.64	—	2.3×10^{-5}	—	43	—	—
2	4.64	—	2.3 "	—	75	—	—
3	4.66	—	2.2 "	—	64	—	—
4	4.67	—	2.15 "	—	74.5	—	—
5	4.68	4.68	2.1 "	2.1×10^{-5}	56	58	40
6	4.68	4.68	2.1 "	2.1 "	71	74.5	60
7	4.70	4.70	2.0 "	2.0 "	73	78.2	30
8	4.73	4.73	1.9 "	1.9 "	73.8	78.2	—
9	4.75	—	1.8 "	—	69	—	—
10	4.78	—	1.7 "	—	58.9	—	—

The data furnished by these experiments are given in Table XI.

Milk begins to coagulate during the process of souring when the pH value reaches 4.64 to 4.78, representing a change of C_H varying from 2.3×10^{-5} to 1.7×10^{-5}. The total acidity by titration varies from 43 to 75 cc. of 0.1 N acid per 100 cc. of milk.

The period of coagulation from beginning to completion varies from 30 to 60 minutes at 25°C. *The hydrogen ion concentration remains constant during the change.* The acidity by titration increases slightly, varying from 2 to 5.2 cc. of 0.1 N acid per 100 cc. of milk.

Relation of First Sign of Souring in Milk to Hydrogen Ion Concentration.

The first detectable sign of souring in milk is popularly thought to be a sour or acid taste or smell. As a matter of fact, that property which one associates with the first or preliminary stage of souring is more accurately characterized as a flavor; that is, a quality perceptible to the two senses of taste and smell. It is a common experience for one, when suspicious of the condition of milk or cream, to smell of it or taste of it and to say that it smells sour or tastes sour even when it does not taste really acid. In the ordinary souring of milk, some volatile compound or compounds, not possessing a sour or acid taste, are regularly formed in minute amounts as preliminary products or by-products of the process of lactic acid formation. Much remains to be learned about the details of the process. As a result of associating the odor and taste of such volatile flavor products with the acid or sour taste of milk, we have formed the habit of saying that milk tastes sour whenever we detect this associated flavor, even though there is no real sour or acid taste.

As a preliminary study, we have made some observations in the case of several samples of milk undergoing the process of souring, in which the milk was examined at intervals of 1 hour or less for the characteristic flavor or so-called sour taste preceding the appearance of actually sour or acid taste. When it was possible in the sample under observation to detect by the taste and smell the first indication of the characteristic flavor, we at once made determination of the hydrogen ion concentration and acidity by titration. The samples numbered 1 to 4 were soured in the ordinary way at 25°C. without addition of organisms; Samples 5 and 6 were pasteurized and then inoculated with a sour-milk "starter" which had been nearly neutralized with dilute alkali.

The results of the work are given in Table XII.

The data in Table XII do not show any relation of the hydrogen ion concentration to the time at which the characteristic flavor indicative of the souring process is first discernible. In no case did the milk taste distinctly acid; and, if the nasal passages were closed, there was no taste at all. The so-called sour flavor could be perceived by the smell alone or by the combined sense

TABLE XII

Hydrogen Ion Concentration in Relation to First Stage of Souring in Milk.

No. of sample.	Time after starting experiment when sample was examined.	0.1 N acid formed in 100 cc. of milk.	Hydrogen ion concentration expressed as	
			pH	C_H
	hrs.	*cc.*		
1	21	37.5	5.44	0.37×10^{-5}
2	34	24.2	5.44	0.37 "
3	28	31.5	5.49	0.33 "
4	24	26.5	5.49	0.33 "
5	20	18.8	5.58	0.26 "
6	21	22.0	5.60	0.25 "
7	6	15.0	5.96	0.11 "

of taste and smell. Solutions containing lactic acid and calcium lactate have a much higher hydrogen ion concentration (about 5.1 pH or C_H, 0.8×10^{-5}) before they taste sour or acid than any in Table XII. Milk to which lactic acid is added fails to develop the flavor characteristic of milk naturally soured. The compound or compounds responsible for the flavor in question is due to bacterial action without doubt.

The results here presented are of value chiefly for their suggestiveness as a basis for further work.

SUMMARY.

1. The work was undertaken to ascertain how much free lactic acid is present in sour milk. Lactic acid exists in sour milk largely as lactate but partly as free acid. Part of the free acid is in solution while a smaller part is adsorbed by the casein. Each must be determined separately. In addition a study was made of the acidity of sour milks, the coagulation point of sour milk, and the first sign of souring.

2. In all experiments the milk was kept at 25°C. during the souring process. Freshly separated skim-milk, pasteurized at 62°C., cooled to 25°C., was inoculated with *Bacterium lactis acidi*. In studying the acidity of sour milks, samples were inoculated with other organisms also.

3. In determining the amount of free lactic acid, three different methods were employed with results in good agreement: (*a*)

Measurement of hydrogen ion concentration, and application of calculation based on the mass law; (*b*) partial extraction by ether and application of calculation based on coefficient of distribution; (*c*) double electrometric titration with lactic acid and hydrochloric acid. Under the conditions of the experiment, free lactic acid does not appear in appreciable amounts in souring milk for about 20 hours after inoculation, when there is present 0.1 cc. of 0.1 N acid in 100 cc. of milk; this increases more rapidly in the next few hours and finally in 48 hours it is about 20 cc. The pH value changes from 6.5 in fresh milk to 4.17 in 48 hours.

4. In estimating the amount of free lactic acid adsorbed by casein in sour milk, four methods were used, giving consistent results: (*a*) Measurement of reduction of hydrogen ion concentration caused by adding casein to lactic acid solutions of given concentration; (*b*) measurement by titration of reduction of acidity caused by adding casein to lactic acid solutions; (*c*) titration of acidity of sour milk and of separated whey; (*d*) extraction of lactic acid in sour milk by ether and in separated whey. About 20 per cent of the free lactic acid in coagulated sour milk is adsorbed by the casein.

5. In making a study of the acidity of milks soured under different conditions, fresh separated milk was soured at 25°C., after preliminary treatment in the following ways: (*a*) Pasteurized milk inoculated with (1) *Bacterium lactis acidi*, (2) with *Bacillus bulgaricus*, (3) *Streptococcus lacticus;* (*b*) unpasteurized milk (1) inoculated with a sour-milk "starter," (2) self-inoculated. The total acidity by titration varied from 70.5 to 220 cc. of 0.1 N acid per 100 cc. of milk; the free lactic acid, from 8.6 to 104 cc.; the acid as lactate, from 51.8 to 92 cc.; the pH value, from 3.70 to 4.56. In milk souring under ordinary conditions, the total acidity by titration varied from 70.5 to 107.5 cc. 0.1 N acid per 100 cc. of milk; the free lactic acid, from 13.1 to 34.5 cc.; the pH value, from 4.02 to 4.43.

6. The casein of milk begins to coagulate when the pH value reaches 4.64 to 4.78. The time from the beginning of coagulation to completion varies from 30 to 60 minutes, during which period the hydrogen ion concentration remains constant, though the acidity by titration increases slightly.

7. The first physically and easily perceptible sign of souring in milk is a characteristic flavor, discernible to the senses of both smell and taste, due to the presence of some volatile compound formed in the souring process and not to lactic acid. The flavor appears before the milk begins to taste acid. There is no apparent relation between either the hydrogen ion concentration or the acidity by titration and the first sign of this flavor.